PURIFICATION AND CHARACTERIZATION OF SOLUBLE β-GALACTOSIDE BINDING LECTIN FROM BUFFALO BRAIN

SUMMARY

OF THE

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

BY

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2008

Summary

SUMMARY

Depending on the requirement of calcium for their hemagglutination activity, the animal lectins have been divided into two major types, the S type (calcium-independent) and C type (calcium-dependent). The S-type lectins are soluble, β-galactoside-specific, evolutionarily conserved proteins widely distributed in nature from lower invertebrates to mammals. In addition, they require the presence of thiol reducing reagents to retain their carbohydrate-binding activity. So far, β-galactoside binding proteins or galectins have been implicated in various fundamental processes such as embryonic development, cell-cell interactions, cell migration, immune regulation and organization of nervous system. Moreover, these proteins are very well expressed in mammalian nervous system but its exact role is still obscure.

β-galactoside binding lectin from buffalo brain was purified to homogeneity by using a combination of 40-70% ammonium sulphate fractionation and gel filtration chromatography on sephadex $G_{50\text{-}80}$ column. The purification resulted in 1716 fold enrichment of specific activity with a yield of 0.04 %. The molecular weight of buffalo brain lectin (BBL) as determined by SDS-PAGE under reducing and non-reducing conditions was 14.5 kDa, however, with gel filtration under native conditions, it was 28.5 kDa, revealing the dimeric form of the protein. The stokes radius calculated from gel filtration data was 25 Å and diffusion coefficient corresponded to 8.91×10^{-15} cm^2/s.

The neutral sugar content of the soluble lectin was estimated to be 3.3 %, whereas thiol analysis indicated the presence of 3 sulphydryl groups per mole of BBL. Alkylation of sulphydryl groups of brain lectin by iodoacetate and iodoacetamide resulted in inactivation of hemagglutinating activity, suggesting the need of reducing agent to maintain BBL in its active form. The specificity of BBL for various saccharides was determined by hemagglutination inhibition assay. BBL displayed affinity for lactose and other sugar moieties in glycosidic form, suggesting it to be a β-galactoside binding lectin. Moreover, BBL preferentially agglutinated trypsinized human type A as well O erythrocytes. The temperature and pH activity profile displayed the maximum hemagglutination activity of BBL between a temperature range of 30-45 °C and a pH range of 7-7.5. Binding parameters of BBL for lactose was determined by equilibrium dialysis, with 6.6×10^3 M^{-1} association constant (K_{ass}) and two binding sites per lectin molecule.

The effect of various denaturing agents like GdnHCl, urea and thiourea on BBL was studied and it was found that BBL considerably lost its activity at 6 M concentration of denaturants. Similarly, SDS also abolished the lectin activity but at very low concentration, while non-ionic detergents like Tween-20 and Triton X-100 exhibited a mild inhibitory behavior against BBL. However, pre-incubation of BBL with lactose largely prevented the detergent induced denaturation of the protein.

The purified lectin was investigated for its brain cell aggregation properties by testing its ability to agglutinate cells isolated from buffalo and goat brains. Rate of aggregation of buffalo brain cells by purified protein was more than the goat brain cells.

The treatment of BBL with oxidizing agent (H_2O_2) abolished its agglutination activity and shifted its UV absorption maxima from 282 to 250 nm, suggesting the oxidation of tryptophan residue present in BBL. In addition, conformational changes in BBL induced by H_2O_2 were also monitored by fluorescence, circular dichroism and FTIR spectroscopy. Fluorescence spectra of BBL in presence of oxidizing agent displayed a quenching in the fluorescence intensity profile, confirming the oxidation of aromatic acid residues. However, the presence of lactose was protective against oxidative structural perturbations as the fluorescence intensity of BBL pre-incubated with lactose displayed a lesser degree of H_2O_2 induced quenching. Similarly, circular dichroism and FTIR analysis revealed that the presence of oxidizing agent completely destroyed the native conformation of BBL. Far UV and FTIR spectra of oxidized BBL suggested the loss of secondary state, thereby changing the β-pleated (native) into α-helical structures, whereas the near-UV spectra confirmed the oxidant induced perturbations in the native tertiary structure of BBL. However, pre-incubation of BBL with lactose did not bring any change in the native conformation of BBL both at secondary and tertiary levels. Moreover, a protective effect of lactose against oxidizing action of H_2O_2 on BBL was also demonstrated by both CD and FTIR spectroscopy.

The purified lectin from buffalo brain revealed the presence of 3.3 % sugar residues. Thus, the possible role of carbohydrate moiety in the stabilization of the protein was investigated by deglycosylating the lectin using periodate oxidation method. Deglycosylated and glycosylated forms of brain lectin were subjected to a comparative analysis using protein activity and fluorescence as probes over a wide range of temperature, pH and in the presence of various detergents and chaotropic

agents. The native form of lectin retained greater fraction of hemagglutinating activity against various physical and chemical denaturants. The unfolding of both the forms of lectin in the presence of GdnHCl, urea and thiourea studied by fluorescence indicated greater perturbations in the conformation of deglycosylated lectin than the native protein. The different properties examined thus indicated that glycosylation plays an important role in the stabilization of native conformation of protein against the inactivation caused by various denaturants.

Antibodies raised against pure BBL gave a single precipitin band with BBL and the titre determined by direct binding ELISA was found to be more than 12800 suggesting the high immunogenic nature of the protein. Moreover, anti-BBL antibodies also cross reacted with purified goat and sheep brain lectins, displaying antigenic relationship between them. Dot blot analysis revealed the presence of similar lectin in lung, heart and liver of buffalo which cross reacted with antibodies raised against buffalo brain lectin.

The effect of soluble β-galactoside specific lectin from buffalo brain on the fragility and permeability of bio-membranes was examined using erythrocyte as model. Osmotic fragility of erythrocytes was considerably enhanced in the presence of BBL. The lytic activity of BBL was temperature, pH and incubation period dependent with maximum activity displayed between 30-40°C, pH 6.4-7.5 and 6 hours, respectively.

Modulation of membrane integrity under oxidative stress in the presence and absence of β-galactoside binding protein was also investigated. Exogenous brain lectin considerably enhanced the susceptibility of erythrocyte membranes to free radical injury and hypochlorous acid induced oxidative assault, indicating the property of lectin to affect membrane dynamics and functions by overall reorganization of membrane components and making it more vulnerable to toxic assaults.

BBL was also used as a diagnostics tool to reveal the expression pattern of β-galactoside residues on the erythrocyte membranes of prostate and breast cancer patients. It was found that breast cancer causes a marked decrease in the expression of β-galactoside residues whereas, no change in the sugar moieties was exhibited in patients with prostate cancer.

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Certificate

*I certify that the work entitled '**Purification and characterization of soluble β-galactoside binding lectin from buffalo brain**', embodied in this thesis is an original work done by Ms. Sabika Rizvi under my supervision and is suitable for the award of Ph.D Degree in Biochemistry.*

(Prof. Naheed Banu)

Dedicated to my loving parents and my little sister

An effort supported by my dearest husband, Hasan

ACKNOWLEDGEMENTS

To start with, I would first bow my head infront of my almighty "Allah" for showering upon me the wealth of knowledge and paving all the ways through every thick and thin, to reach the zenith of my academic career.

Its simply next to impossible to pen down my gratitude towards my supervisor, my esteemed teacher and my mother figure, Prof. Naheed Banu who undoubtedly went out of her way to guide, support and advise me throughout in achieving this milestone of my career. She stood like a rock against every tidal wave so that I could transform my dreams into reality. 'Thanks a lot, Mam...Without you I would not have got an opportunity to write this acknowledgement'.

I am highly grateful to Prof. S.M. Hadi, Chairman, Department of Biochemistry, for providing necessary facilities for research work.

I would also like to thank Prof. Masood Ahmed, Prof. Qayuum Husain, Prof. Bilqees Bano, Prof. A.N.K. Yusufi, Dr. Faheem Halim Khan, Dr. Tabish, Dr. Imrana Naseem and all other teachers for their precious helping hands.

This thesis is a cumulative effort of many people who are very precious and indispensable part of my life. First and foremost, my heartiest thanks to Toshiba, who as a roommate, a classmate and a sister alike friend, always stood like a pillar of strength to support me in times of need and distress. Inspite of going through a very crucial stage of her life she was always a bundle of encouragement and inspiration for me.

If one person is to be named without whom this thesis would have been impossible, then he is none other than Mohd. Shahnawaz Khan, whose support strength, confidence, and morale boosters proved like lampposts for me on the dark streets of my research world. His everhelping attitude, caring nature and his encouraging one liner, 'Ho jaega Apa....' were definitely stress relievers during times of depression and frustration.

My acknowledgement would be incomplete if I do not mention a note of appreciation for Shams who went beyond my expectations to stand beside me and lend his helping hands during the compilation of this thesis.

My heartfelt thanks to the immaculate support lend by Aliya, Sarmad, Sadia Apa, Medha and Ayesha Apa who were always there to share my moments of joy as well as sorrows. I would also like to thank Ashraf, Nida, Bilaal, Atif, Mahreen, Rukhsana for their generous help and cooperation during difficult moments.

This thesis is undoubtedly a dream for four pillars of my life. First two are definitely my parents for whom no phrase or no word can express my thanks. They always stood like a beam of sunshine while I was climbing the stairs of my academic career. The best thing about them was inspite they knew very little about my actual work, still they left no stones unturned to help in times of need. 'May every child is blessed with such lovely parents as mine'.

If it's my parents who taught me to dream about my career, it was my husband who turned these dreams into a reality. His support, encouragement and confidence in me were simply marvellous and commendable. Hats of to his patience and grace, for allowing me to complete my Ph.D at the cost of his newly wedded life and enduring my absence from home. I completely owe the finishing point of my career solely to him. 'Thanks a lot Hasan.... for being such a wonderful life companion'.

Last but definitely not the least is my best friend, my replica, and my dearest sister Aman whose moral and emotional support proved to be a guiding light through the dark gallows of research.

Finally, a note of thanks to each and every person who was directly or indirectly involved in this research work.

'Thanks a lot everyone.....for making me reach this landmark of my life'.

Sabika Rizvi

(Sabika Rizvi)

CONTENTS

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromole
ALS	Amyotrophic lateral sclerosis
Ar	Arginine
Asp	Aspartate
BBL	Buffalo brain lectin
BM	Bone marrow
C type	Ca^{2+} dependent
CD	Circular diochroism
CD-MPR	Cation-dependent mannose 6-phosphate receptor
CNS	Central nervous system
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
CM	Carboxymethyl
Cys	Cysteine
DRG	Dorsal root ganglion
DEAE	Di-ethyl-amino-ethyl
DTNB	5-5'-Dithiobis 2- nitrobenzoic acid
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FGFR	Fibroblast growth factor receptors
FTIR	Fluorescence tagged infrared spectroscopy
GAG	Glycosaminoglycans
GAL	Galactose
GBM	Glomerular basement membrane
GdnHCl	Guanidine hydrochloride
GlcNAc/	Glucose -N-acetyl
Glu	Glucose
GRIFIN	Galectin related interfiber protein
H_2O_2	Hydrogen Peroxide

HIV	Human immuno deficiency virus
HOCl	Hypochlorous acid
hr	Hour
ICAM	Intracellular adhesion molecules
IGF-II/MPR	Insulin-like growth factor II/mannose 6-phosphate receptor
MAG	Myelin associated glycoprotein
mg	Milligram
ml	Milliliter
mM	Millimole
MPR	Mannose 6-phosphate receptor
NaOCl	Sodium hypochlorite
NaCl	Sodium chloride
NCAM	Neural cell adhesion molecules
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCTA-1	Prostate carcinoma tumor antigen-1
PNS	Peripheral nervous system
RBC	Red blood cell
SAP	Serum amyloid P component
SDS	Sodium dodecyl sulphate
S-type lectin	Sulphydryl dependent
TNF	Tumor necrosis factor
Trp	Tryptophan
Tr	Trophectoderm
v/v	Volume/Volume
w/v	Weight/volume

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Preface

PREFACE

Animal lectins are a unique class of proteins generally defined as non-enzymatic, non-immunoglobulin molecules recognizing specific carbohydrate structures. This category of sugar binding proteins is widespread in all taxa of the animal world ranging from nematodes to human beings, where they are involved in a number of fundamental processes. On the basis of location, vertebrates possess two types of lectins, one is membrane integrated lectin apparently involved in translocation of glycoconjugates in cells and the other is soluble lectin that plays a role in the secretion or organization of extracellular glycoconjugates. Depending upon their metal requirement nature, lectins belonging to kingdom animalia can be classified into C-type (Ca^{2+} dependent) and S-type lectins (sulphydryl dependent). Soluble β-galactoside binding lectins are a category of metal independent S-type lectins that binds specifically to β-galactoside residues present in cellular glycoproteins and glycolipids. Recent nomenclature classify these β-galactoside binding proteins as 'galectins' which are defined as highly evolutionarily conserved family of proteins that share structural similarities in the carbohydrate recognition domain in addition to specificity for polylactosamine-enriched glycoconjugates. They are further classified on the basis of subunit structure into prototype (single domain), tandem repeat type (two homologous domains) and chimera type (galectin domain combined with unrelated protein domain).

The localization of β-galactoside lectins is generally varied ranging from inside to outside the cells. In the former case, both the cytoplasm and nucleus are the sites of location, whereas in the latter case, they are either attached to the cell surface or localized in the intracellular spaces between closely packed cells. Thus, galectins have distinct biological roles depending on their sites of location. The versatility of lectins in animal tissues and their capacity for multiple interactions with carbohydrates as well as non-carbohydrate ligands, makes them potentially important factors for the interaction of cells with their environment. For example, galectins play a key role in linking extracellular and intercellular signals to control survival and motility in lymphocytes and epithelial cells (Hernandez and Baum, 2002; Rabinovich et al., 2002; Stillman et al., 2005; Elola et al., 2007). In addition, role of galectins in normal epithelial and hematopoietic development, as well as in the epithelial carcinogenesis and lymphomagenesis, have been clearly demonstrated by several workers

(Hughes, 2001; Hoyer et al., 2004; Hasan et al., 2007). They also regulate cell cycle progression and apoptosis, serve as adhesion and deadhesion molecules and are actively involved in RNA splicing (Allione et al., 1998; Kim et al., 1999; Cooper, 2002; Rabinovich and Gruppi, 2005). Precisely, they serve an overwhelming variety of essential physiological and pathological functions including tumorigenesis, cancer progression and metastasis.

Galectins have been isolated and studied from such diverse sources starting from electric eel (Levi and Teichberg, 1981), amphibians (Bols et al., 1986), reptiles (Gartner et al., 1980), aves (Beyer et al., 1980) and mammals (Harrison et al., 1984), expressed in an array of different tissues like skin, muscles, heart, lungs, kidney, intestines and nervous system carrying out diverse activities owing to their multivalent sugar binding property. The presence of galectins in mammalian nervous system has been reported by several workers, which are expressed in a sub-population of dorsal root ganglion neurons, astrocytes, perivascular cells and microvessels (Sango et al., 2004; Stillman et al., 2005; Mok et al., 2007) and form the neural network of the olfactory bulb (Puche et al., 1996; John and Key, 1999; Imbe et al., 2003; Horie et al., 2004). Although, various function of soluble lectin in mammalian nervous system in terms of physiological and pathological parameters have been suggested, but its exact role is still obscure. However, recent findings suggest its involvement in stimulation of axonal regeneration, neurite outgrowth, synaptic connectivity and establishment of neuropathic pain after the peripheral nerve injury (Imbe et al., 2003; Horie et al., 2004; Stillman et al., 2005; Stillman et al., 2006). To add to the list of roles played by galectin in mammalian nervous system, latest inclusions are its neuroprotective and therapeutic effect during amyotrophic lateral sclerosis (Chang-Hong et al., 2005), neurodegenerating effect in scrapie infected central nervous system (Mok et al., 2007) and a pleiotropic role during glioblastoma cell motility (Debray et al., 2004).

β-galactoside binding lectins have been previously purified and characterized from brain tissues of various mammals like rat, cow, human, goat and sheep (Caron et al., 1987; Bladier et al., 1989; Ola et al., 2001; Shahwan et al., 2004). In view of the fact that the purification of this protein has not been reported from buffalo brain and its physicochemical properties have not been fully investigated, we have done a detailed study of this soluble lectin, hoping that the intimate knowledge of this protein may help in understanding its physiological functions in mammalian nervous system.

Moreover, buffalo brain has been used as it is a cheaper and easily available source for investigation of lectins from mammalian nervous system.

In the present study lectin having an affinity for β-galactosides has been purified from water buffalo (*Bubalus bubalis*) brain by a combination of ammonium sulphate precipitation and gel permeation technique. A detailed study of physicochemical properties and the effect of various inactivating agents on the functional and structural aspects of purified brain lectin have been sketched down. Till date, all the mammalian brain lectin (Caron et al., 1987; Bladier et al., 1989; Ola et al., 2001) isolated are found to be devoid of any carbohydrate moiety. Therefore, it was interesting to explore the glycosylated nature of the purified protein and study the role of glycosylation on the structure and functions of lectin by a co-operative analysis of its activity and structural stability in both glycosylated and deglycosylated forms over a wide range of temperature, pH, detergents and in the presence of various denaturing agents.

Since these lectins remain in active conformation in the presence of a reducing agent, it led us to believe that they contain an oxidizable residue whose integrity is quite crucial for its activity. Therefore, a detailed evaluation of modification of brain lectin in the presence of H_2O_2 in terms of functional and structural parameters has been undertaken.

Moreover, immunological studies have also been carried out to elucidate the antigenic relationship of buffalo brain lectin (BBL) with other similar brain lectins of different species and the presence of same lectin in different organs of buffalo by dot blot assay.

The hemolytic and cytolytic action of purified lectin has been studied by monitoring its effect on erythrocytes cell membrane integrity and permeability. This would shed light on the role of lectin in cytolytic destruction of foreign cells in mammalian nervous system.

Since erythrocytes of various carcinoma cells show a distinct pattern of glycosylation which becomes a diagnostic index to examine the presence and proliferation of well known cancers, we also intended to study the presence and absence of β-galactoside sugar residues on erythrocyte membrane of different cancer patients using brain lectin as a tool. To sum up, the objectives and the overall aim of the present study is to contribute to the existing repertoire of the diversity, structure and biological role of β-galactoside binding lectins in mammalian nervous system.

Review of Literature

REVIEW OF LITERATURE

Milestones in discovery of lectins

The term lectin (latin word means 'legre' or 'to choose') was first coined by Boyd and Shapleigh (1954) to describe a class of proteins of plant origin which agglutinates cells and exhibit antibody like sugar binding specificity. However, the existence of lectin in biological materials especially in plant seeds can be traced far back into history with the discovery of ricin (Tschrich, 1912; Olsnes and Phil, 1982) and abrin (Tschrich, 1925; Olsnes and Phil, 1982). Infact, the original lectin saga begins in 1888 with the finding of Herman Stillmark during his doctoral research in Kobert's laboratory where he partially isolated proteinacious preparations called 'ricin' from castor beans (Barondes, 1997). His work initiated a series of discoveries that unveiled the presence of lectins in all spheres of biological kingdoms including virus, fungi, lichens, chordates, invertebrates and vertebrates. **Animal lectins**, including mammalian lectins, have been discovered much before than plant lectins, although the contexts of their discoveries were often rather different and some were discovered long before their identification as carbohydrate binding proteins (Kilpatrick, 2002). The first animal lectin observed was probably found in snakes when Flexner and Noguchi (1902), discovered the agglutinating and lytic activity of snake venoms. Infact, this idea was originally prompted by S. Weir Mitchell in 1886, two years before the Stillmark's discovery of plant lectin activity, so we may conclude that Mitchell was the first researcher to observe animal lectin activity and probably the first to observe lectin activity per se, whether of plant and animal origin (Kilpatrick, 2002). Later, Stockert et al., (1974) discovered the first mammalian lectin from rabbit liver which was a major milestone in the history of lectin research in animal kingdom. Apart from liver, lectins were also isolated and purified from electric organ of electric eel (Levi and Teichberg, 1981), calf heart and lungs (De Waard et al., 1976). These lectins are dimers with subunit molecular weight of 14,000 dalton, requires reducing agents for their activity and bear specificity for β-galactoside binding. Later years till date witnessed the discovery of innumerable specific lectins from a variety of novel animal sources expressed in a wide array of different tissues ranging from muscle (Nowak et al., 1977) through brain (Kobiler and Barondes, 1977; Caron et al., 1987; Bladier et al., 1989; Ola et al., 2001; Shahwan et al., 2004), pancreas (Beyer et al.,1979), colon (Schoeppner, 1995), intestines (Tardy et al., 1995), retina (Uehara et al., 2001) , skin (Marschal et al.,1992) , serum (Colley et al.,1988), placenta

(Visegrady et al., 2001; Than et al., 2004), oocytes (Perillo et al., 1998; Rabinovich, 1999) and uterus (Gray et al., 2004).

Classification of animal lectins

On the basis of the primary structure of animal lectins, Drickamer classified animal lectins into two structural families: the C-type (requiring Ca^{2+} for activity) lectins and S-type (sulfydryl-dependent or β-galactoside binding) lectins (Drickamer, 1988). Both families had a conserved carbohydrate recognition (CRD) domain of approximately 130 amino acids residues, although the C-type and S-type domains are completely unrelated to each other. The few exceptions known at that time were a heterogenous group referred to as N type (not C or S) (Kilpatrick, 2002). However, the present day perception is very different with vastly more structural knowledge available (Gabius et al., 2002, Loris et al., 2002; Ahmed and Vasta, 2008), it is possible to list at least 15 such families in addition to the C-type and S-type (Table I). Out of these 15 families only five major classes have been discussed below.

C-type lectins

C-type lectins are the most diverse family of animal lectins. These lectins are generally multidomain proteins, in which C-type homologous CRDs provide Ca^{2+} dependent sugar recognition activity and a variety of other modules initiates a broad range of biological activities such as cell adhesion, endocytosis and pathogen neutralization (Dodd and Drickamer, 2001). The sugar binding sites in vertebrates C-type CRDs are formed in part by a bound Ca^{2+} which must be present for the sugar binding to occur (Dodd and Drickamer, 2001). The majority of C-type lectins bind to D- mannose, D-glucose and related sugars (Man-type ligands), or to D-galactose and its derivatives (Gal-type ligands) (Kolatkar and Weis, 1996). C type lectins with high affinity for glycoconjugates bearing terminal galactose residues have been identified on the surfaces of peritoneal macrophages, Kupffer cells (Hoyle et al., 1988; Ii et al., 1990) and appear to mediate recognition of tumor cells (Sato et al., 1992), whereas C-lectins with lower affinity for Gal-type ligands are found in proteoglycan core proteins of cartilage and other tissues and are presumed to contribute to the organization of the extracellular matrix (Drickamer and Taylor, 1993). The genuine C-type lectins are more varied than thought and can be classified into subgroups e.g. Hyalectans, asialoglycoprotein receptor, collectins, selectins, transmembrane receptors, macrophage mannose receptor and single domain lectins (Kilpatrick, 2002),

Table I

The different families of animal lectin with known three dimensional structures

Family	Carbohydrate specificity range	Fold
Galectins	Strict galactose/lactose	Beta sandwich
Pentraxins	Variable, often non carbohydrates	Beta sandwich
C-type lectins	Highly variable	C-type lectin
I-type lectins	Sialic acid	Immunoglobulin
P-type lectins	Mannose -6-phophate	M6P- β-sandwich
Cys-MR and FGF2	Sulfated carbohydrates	- β-trefoil
Tachylectin	GlcNAc/GalNAc	Five-bladed – β-propeller
Ym1	Heparin/heparin sulfate	Chitinase
HGF/SF(NK1)	Heparin/heparin sulfate	Unique
Lectin from spider toxin	Heparin	Unique
Cobra venom cardiotoxin	Mannosamine	Hevein
Spermadesins	Heparin, often non carbohydrate	CUB
TNF	Chitobiose	TNF – β-sandwich
Calnexins	Glucose	- β-sandwich
ERGIC-53	Mannose	- β-sandwich

depending upon gene structure and the nature of additional non-lectin domain. Wide range of functions carried out by these lectins include catabolism of partially degraded glycoproteins containing terminal galactose or N-acetylgalactosamine residues (Schwartz, 1984), clearance of galactose terminal glycoproteins, desialyted erythrocytes and other blood cells (Sharon and Lis, 1989), cell-cell interaction during spermatogenesis and fertilization (Goluboff et al., 1995), targeting of hydrolytic enzymes to lysosomes (Sahagian, 1984), regulation of blood clotting (Stockert, 1995) and as a protein that guides leukocytes (Crottet et al., 1996) and also plays major roles in chronic rejection process of transplanted tissues (Russell et al., 1994).

P-type (phosphomannosyl receptors)

P-type lectins are mannose-6-phosphate recognizing, calcium independent group of lectins which have unique repeating motif in the carbohydrate recognition domain (Dahms, 1996). These lectins play an essential role in the generation of functional lysosomes within the cells of higher eukaryotes by directing newly synthesized lysosomal enzymes bearing the mannose 6-phosphate signal to lysosomes (Dahms and Hancock, 2002). The two members of the P-type lectin family, the cation-dependent mannose 6-phosphate receptor (CD-MPR) and the insulin-like growth factor II/mannose 6-phosphate receptor (IGF-II/MPR) are distinguished from all other lectins by their ability to recognize phosphorylated mannose residues (Drickamer and Taylor, 1993). Although the reported subcellular distribution of the P type lectins varies among cell types (Waguri et al., 2001), in general about 10% of the receptors are present at the cell surface while the remainder of the MPRs are found predominantly in late endosomal compartments and the trans golgi network. The ability of the IGF-II/MPR to recognize many functionally distinct ligands illustrates the multifunctional nature of this receptor and its involvement in a myriad of important physiological pathways including its crucial role in mammalian growth, including heart development by influencing fetal cell division and differentiation (Dell and Day, 1998). Over-expression of IGF-II is observed in various human cancers (Ellis et al., 1998), overgrowth syndromes (Sperandeo et al., 2000) and development of atherosclerosis (Zaina et al., 2002), thus suggesting its role in various pathological conditions.

I- type lectins

I-type lectin is a collective term introduced by Powell and Varki (1995) to describe carbohydrate-recognizing proteins that belong to the immunoglobulin (Ig)

superfamily. According to their specificity and binding affinities, these lectins are classified into many subclasses. Thus, there are I-type proteins recognizing sialic acids, other sugars and glycosaminoglycans. Among the I-type lectins recognizing sialic acids are the Siglecs (Sialic acid-binding immunoglobulin superfamily lectins), CD-83 (Scholler et al., 2001) and cell adhesion molecule L1 (Kleene et al., 2001).

Each Siglec has a distinct expression pattern in different cell types, indicating that they perform highly specific functions including critical roles in neural development, such as neural cell adhesion, positive and/or negative regulation of neurite outgrowth and myelin sheath formation (Filbin, 1995).

CD-83 is a 45 kDa glycoprotein expressed on mature dendritic cells (Zhou and Tedder, 1995), thus suggesting its involvement in the interaction between dendritic cells and circulating monocytes as well as activated and/or stressed T cells.

Cell adhesion molecule L1 is a homophilic and heterophilic adhesion molecule of 200 kDa found in the nervous system, $CD4^+$ T cells, monocytes and B cells (Ebeling et al., 1996) where it plays an important role in axon guidance, cell migration and neurogenesis (Angata and Linden, 2002).

I-type lectins bearing specificity towards other sugars than sialic acids includes neural cell adhesion molecules (NCAM), Myelin Protein Zero (P_0), ICAM-1/CD54 (Intracellular adhesion molecules), CD 2 and hemolin. They are present in a wide array of tissues including nervous system, vascular endothelium, certain lymphocytes and monocytes where they are involved in T cell development and myelination of peripheral neurons (Warner et al., 1996), inflammation and wound healing (Angata and Linden, 2002). I-type proteins interacting with sulfated glycosaminoglycans (GAGs) show selectivity towards the type of GAGs they interact with (in many cases heparin/heparan sulfate are the favored ligands). This category includes vertebrate fibroblast growth factor receptors (FGFR), Perlecan (heparan sulfate proteoglycan-2/HSPG2) and CD48 and are essential to mammalian embryonic development (Arikawa et al., 2001) and $CD4^+$ T cell activation (Hopf et al., 2001).

Pentraxins

The term 'pentraxins' was applied to C-reactive proteins (CRP) and its homologue, serum amyloid P component (SAP), to reflect their unusual quarternary structure in which five identical polypeptide subunits combine to form a ring with a central hole, loosely resembling a doughnut (Kilpatrick, 2002). These proteins are complement activating molecule with high affinity for phosphorylcholine, however, CRP has also

been found to bind to galactans and galactose phosphate but using a different binding site (Kilpatrick, 2002). Pentraxins are expressed in vertebrate species, where the division into CRP or SAP is not made on the basis of primary structural homology to the human examples, but rather on a preference for phosphorylcholine (CRP) or phosphoethanolamine (SAP) (Iwaki et al., 1999; Kilpatrick, 2002).

S-Type lectins or β-galactoside binding lectins

S-type lectins refer to soluble vertebrate tissue lectins which require thiol reducing agents to maintain their activity. However, this property is not shared by all of the S-type lectins but specificity towards β-galactosides is a common characteristic among this class of lectins, therefore a consensus was reached that the term 'β-galactoside binding' or galectin would be more apt for the nomenclature of these specific proteins (Kilpatrick, 2002). In 1973, β-galactoside binding lectins were first discovered in *Dictyostellium discoidium*, a popular slime mould species during the study of eukaryotic development and differentiation, which was inhibited by lactose, galactose and related sachharides (Kilpatrick, 2002). This agglutinin, first named discoidin, then discoidin-1, exhibited a 400-fold increase in specific activity during aggregation of amoebae and was originally thought to mediate the adhesive contacts between the cells. Within a few years, several rather similar lectins were discovered in related or indeed the same (discoidin) species (Barondes, 1986). All were readily inhibited by lactose, but differed in fine specificity, including relative affinities for galactose and N-acetylgalactosamine. An apparently similar lactose specific was reported from the electric organ tissue of the electric eel (Teichberg et al., 1975). The discovery of electrolectin prompted the researchers to survey various tissues of higher animals for lactose specific agglutinins. Such activity was found to be very widely distributed amongst the numerous rat organs examined, but the highest specific activity was found in pectoral muscle of chicken embryos. All the lectins isolated were dimers exhibiting subunit molecular weights of about 15 kDa with the exception for intestine lectin (subunit molecular weight 14 kDa) which behaved as monomer and had a somewhat different carbohydrate binding site. Later soluble lectins were isolated from several mammalian tissues including human heart (Child and Feizi, 1979), muscles (Child and Feizi, 1979) as well as rabbit bone marrow (Harrison et al., 1984). Thus, it was apparent that these 'soluble lectins', 'endogenous lectins, 'galaptins' or β-galactoside-binding proteins occurred universally in vertebrate tissue (Harrison, 1991). Many of the vertebrate lectins are structurally homologous;

however, the term **"galectin"** is now preferred to denote members of this family (Kilpatrick, 2002). Recent survey of literature shows that work on galectin is not only limited to purification and characterization, but has rather spread to more wide arrays like artificial synthesizing galectin using recombinant DNA technique (Timoshenko et al., 2003; Leffler et al., 2004; Ahmed and Vasta, 2008; Nagae et al., 2008). Studies on galectin are mainly related to their functional roles and their structural changes upon binding to sugar residues (Patnaik et al., 2006; Mok et al., 2007). Analysis of Genebank databases has led to the identification of more galectin-like proteins in mammals, invertebrates, plants and microorganisms, confirming that these carbohydrate binding proteins are highly conserved throughout the evolution (Cooper 2002; Leffler et al., 2004). Almost of them appear in human genomic DNA and their mRNA is also expressed, suggesting that they are not pseudogenes (Cooper and Barondes, 1999; Leffler et al., 2004). As sequence databases continue to explode, many more galectin relatives will be discovered and fill the gaps in the galectin family tree. In fact, soon, genomics and proteomics may advance to the point that specific interactions of galectin subunits with each other, carbohydrate ligands, or other proteins could be accurately predicted from sequence alone (Cooper, 2002). Most galectins have been proposed to exert discrete biologic effects, according to subcellular compartmentalization, developmentally regulated expression and cell activation status (Zuniga et al., 2001). The wealth of new information promises a future scenario in which galectins or their antagonists will be targeted in respect to their fine structure and detailed mechanism of crucial roles played by them in every aspect of living system.

Structural diversity of galectins

All galectins contain conserved carbohydrate-recognition domains that are responsible for carbohydrate binding (Ahmed and Vasta, 1994; Ahmed et al., 2004; Ahmed and Vasta, 2008). Till date, 15 different galectins (galectin-1 to -15) have been identified (Table II) in mammals (Houzelstein et al., 2004; Ahmed and Vasta, 2008; Nagae et al., 2008) based on different sequence homology of CRDs. Overall structures of Gal-1, -2, -3 and -4 is shown in Fig. 1.

Galectin -1

Galectin-1 is a homodimer protein composed of two non-covalently linked 14-kDa subunits with one carbohydrate recognition domain of 134 amino acids (Houzelstein et al., 2004; Kiss et al., 2007). Since each dimeric molecule possesses two galactoside

binding sites, galectin-1 can mediate either intramolecular or intermolecular crosslinking by binding to more than one sugar residue (Gabius, 2002; Kilpatrick, 2002; Imbe et al., 2003). This protein is found on the cell surface and the extracellular matrix, as well as in the cytoplasm and the nucleus of the cells in various tissues, including skin, muscle, lymph node, dorsal root ganglion (DRG), thymus, lung, spleen and placenta (Imbe et al., 2003; Leffler et al., 2004). Expression of galectin-1 has been also identified in immune privileged sites such as placenta and cornea, suggesting an important role in generating and maintaining immune tolerance (Ilarregui et al., 2005; Rabinovich and Gruppi, 2005; Rabinovich et al., 2007). In-vitro gal-1 induces cell cycle arrest and apoptosis of activated T cells or T cell lines (Matarrese et al., 2005; Ion et al., 2005; Ion et al., 2006). However, the in-vivo functions of gal-1 are currently unclear because targeted disruption of the gal-1 gene in null mutant mice results in the absence of major phenotypic abnormalities, perhaps because of compensation by other family members (Kiss et al., 2007). Moreover, it has been also shown that Gal-1 inhibits chemotaxis and trans-endothelial migration of polymorphonuclear leukocytes in in-vitro studies (La et al., 2003). Expression of Gal-1 in bone marrow-derived mesenchymal (stromal) cells has been implicated in bone marrow (BM) cell differentiation (Panepucci, 2004; Kadri et al., 2005). Gal-1 has also been shown to be a positive growth regulator towards other cell types, such as vascular endothelial cells (Sanford et al., 1990; Rabinovich et al., 2002). It has been speculated that growth inhibitory or stimulatory properties of this lectin are highly dependent on the cell types, cell activation status and concomitant environmental signals (Rabinovich et al., 2002; Rabinovich and Gruppi, 2005). In addition, these effects might be regulated alternatively by the relative levels of Gal-1 in the extra cellular milieu and the equilibrium between its monomeric and dimeric forms, because dimeric gal-1 is required to induce some, but not all, of the biological effects mediated by this carbohydrate-binding protein (Rabinovich et al., 2002, Rabinovich et al., 2007).

Galectin-2

Galectin-2 is structurally closely related to galectin-1 (non-covalent dimer with subunits of about 14 kDa), but has a distinct and a restricted expression profile (Sturm et al., 2004; Rabinovich et al., 2007). Structurally, Gal-2 shares 43% amino acid sequence identity with Gal-1 and the analysis of expression in rat tissues and human tumor cell lines had revealed its presence to be confined to the gastrointestinal tract

Table II
Characteristics and functions of mammalian β-galactoside binding lectin family.

	Type	Tissue distribution	Functions
Galectin-1	Proto	Ubiquitous expression	Apoptosis induction in activated T cells Cell growth, mRNA splicing Regeneration of nerve axon (oxidized galectin-1)
Galectin-2	Proto	Small intestine, stomach	Aberrant neurite outgrowth of the olfactory neuron Risk factor of myocardial infarction
Galectin-3	Chimera	Ubiquitous expression	cell adhesion, mRNA splicing Inhibition of T cell apoptosis Macrophage chemotactic factor AGE receptor Reduced intraperitoneal inflammatory response Accelerated progression of diabetic nephropathy
Galectin-4	Tandem	Gastrointestinal tract	Activation of intestinal $CD4^+$ T cells
Galectin-5	Proto	Erythrocytes (rat)	Erythroblast maturation
Galectin-6	Tandem	Gastrointestinal tract (mouse)	High homology to the C-terminal CRD of galectin-9 High homology to galectin-4
Galectin-7	Proto	Keratinocytes (stratified epithelium)	Marker of all subtypes of keratinocytes
Galectin-8	Tandem	Ubiquitous expression	Cell adhesion Regulation of neutrophil function
Galectin-9	Tandem	Immune cells, lung, gastrointestinal tract	Apoptosis induction in activated T cells Eosinophil chemotactic factor Apoptosis induction in cancer cells Cell adhesion
Galectin-10	Proto	Eosinophils, basophils	Charcot-Leyden crystal Affinity for mannose
Galectin11	Proto	Eye lens	Also called GRIFIN, lacks affinity for β-galactosides,
Galectin-12	Tandem	Adipose tissue	Apoptosis induction in adipocytes
Galectin-13	Proto	Placenta	Pregnancy-related protein High homology to galectin-10
Galectin-14	Proto	Eosinophils (sheep)	Possible involvement in allergic reaction Relatively high homology to the N-terminal CRD of galectin-9
Galectin-15/ OVGAL11	Proto	Sheep stomach with parasitic infection Uterus	Endometrial interactions, uterine immune and inflammatory responses and placental morphogenesis.

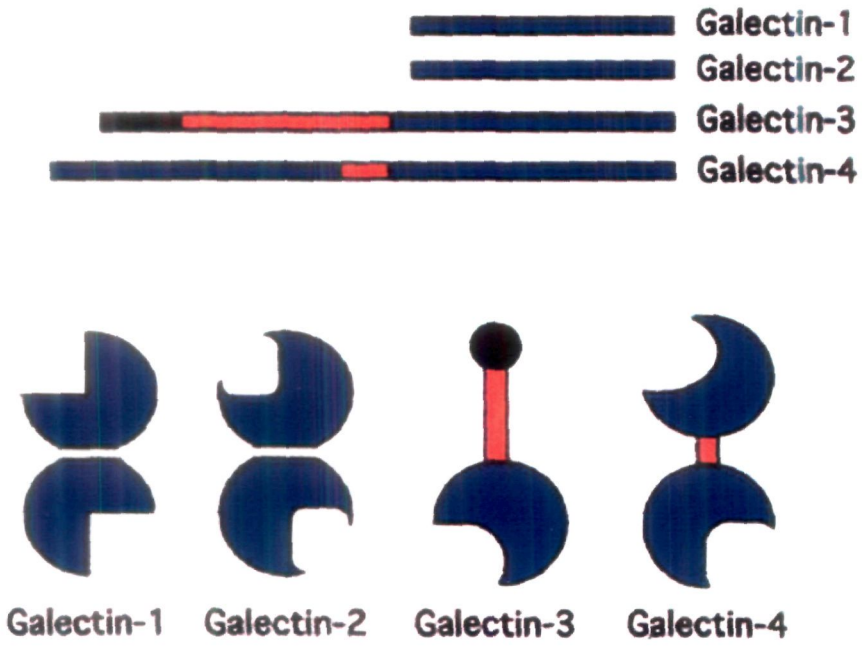


Figure 1. Schematic representation of the overall structures of galectin-1, -2, -3 and-4.

The proteins are shown schematically as linear diagrams corresponding to single peptide chains (top) and as assembled proteins (bottom). The carbohydrate- binding domains of about 130 amino acid residues are blue, the proline-, glycine- and tyrosine-rich repeating domain of galectin-3 (about 100 residues) and link peptide of galectin-4 (about 30 residues) are orange and the N-terminal domain of galectin-3 (about 30 residues) is black. (Barondes et al., 1994)

(Lahm, 2001; Ahmed and Vasta, 2008). Thus, Gal-2 is likely to encounter T cells, especially in inflammatory bowel disease, shedding light on its immunomodulatory capacity (Lahm et al., 2004; Sturm et al., 2004). In contrast to Gal-1, it lacks reactivity towards CD3 and CD7. However, it is a potent inducer of apoptosis of activated T cells, probably mediated by binding to β_1 integrin (a closely associated glycoprotein) (Lahm et al., 2004; Sturm et al., 2004).

Galectin-3

Galectin-3 is a 29 kDa- 35kDa member of the galectin family which promotes cell growth and proliferation and acts as a mitogenic signal towards several types (Liu et al., 2002; Bozice et al., 2004; Ahmed and Vasta, 2008). Galectin-3 consists of an N-terminal domain (about 130 amino acids) made of tandem repeats of short stretches of amino acids connected to a C-terminal carbohydrate recognition domain (Dumic et al., 2006). It is ubiquitously expressed in a wide range of tissues and by inflammatory cells like neutrophils, dendritic cells, macrophages and monocytes (Liu et al., 1995; Dietz et al., 2000; Joo et al., 2001; Acosta et al., 2004). In the peripheral and central nervous system, galectin-3 has been expressed in neurons, Schwann cells and astrocytes including gliomas and microglia (Yang et al., 2006). Gene expression profiling and immunohistochemical analysis has identified galectin-3 expression as highly up regulated in prion-infected brain tissue on the mRNA and protein level (Reimer et al., 2004; Mok et al., 2006), thus suggesting its role in chronic neurodegeneration in prion infected nervous tissue (Mok et al., 2007). Galectin-3 has been implicated in a variety of inflammatory conditions mostly involving innate immune reactions (Bernandes et al., 2006). Extra cellular galectin-3 can also function as an inducer of T-cell apoptosis through binding to CD7 and CD29 on the T cell surface (Fukumori et al., 2003; Fukumori et al., 2004), as well as a positive or a negative growth factor, depending on the target cells (Marer, 2000). In addition, galectin-3 plays a critical role in host protection against infection (Kleshchenko et al., 2004; Vray et al., 2004), for example it displays a direct fungicidal activity by inducing death of *Candida* species containing specific β-1-2 linked oligomannans (Kohatsu et al., 2006) and also binds to wide variety of mammalian pathogens (Beatty et al., 2002; John et al., 2002; Pelletier and Sato, 2002; van der Berg et al., 2004; Levroney et al., 2005; Ouellet et al., 2005; Barboni et al., 2005). Galectin-3 is also strongly expressed in wide variety of cancers (Danguy et al., 2002; Liu et al., 2002; Plazk et al., 2004), thus implicating its role in prognosis and malignancy of tumors

and also inhibits apoptosis in response to chemotherapeutic drugs (Plazk et al., 2004; Debray et al., 2004; Takenaka et al., 2004; Fukomori et al., 2007).

Other galectins

Galectin-4 was the first mammalian lectin consisting of a single polypeptide chain that forms two distinct but homologous CRDs, separated by an un-conserved linker sequence of upto 70 amino acids (Dumic et al., 2006). Thus two CRDs type galectins can bind two individual carbohydrate epitopes. This galectin is a monomer with molecular weight of 36 kDa and is abundantly present in intestinal epithelium (Barondes et al., 1994).

Gal-5 was first discovered as a lactose binding protein isolated from rat lung (Cerra et al., 1985) and erythrocytes (Rabinovich et al., 2007) which consists of one CRD with little additional sequence and has upto 40% identity with galectin-3 and -4 (Leffler et al., 2004; Ahmed and Vasta, 2008).

Galectin-6 has two CRDs that are about 80% identical with those of galectin-4, but the link peptide joining them is 24 amino acids shorter than galectin-4 (Gitt et al., 1998). Galectin -4 and -6 are so similar that they cannot be distinguished in most localization assays such as immunohistochemical and northern blots, but their combined expression can be easily detected and distinguished from other proteins. Possibly galectin-6 is also expressed in small intestine of several mammalian species (Gitt et al., 1997; Leffler et al., 2004).

Gal-7 is a 14 kDa member of the lectin family present in stratified epithelia, interfollicular epidermis and the outer root sheath of the hair follicle (Magnaldo et al., 1995; Leonidas et al., 1998). Thus, galectin-7 is considered as a marker of all subtypes of keratinocytes and its expression does not seem to be influenced by the stages of differentiation (Magnaldo et al., 1995; Bernerd et al., 1999; Saussez and Kiss, 2006). In addition, galectin-7 is also reported to increase the efficacy of chemotherapeutic drugs in urothelial cancer mediated via intracellular reactive oxygen species (Matsui et al., 2007).

Galectin -8 also known as prostate carcinoma tumor antigen 1 (PCTA-1) is a 35 kDa protein made up of tandem repeat CRDs joined by link peptide ((Bidon-Wagner and Le Pennec, 2004) and structurally related to galectin-4 (34% identity). However, unlike galectin-4, which is confined to the intestine and stomach, gal-8 is expressed in liver, kidney, cardiac muscle, lung and brain (Hadari et al., 1995, Hadari et al., 2000). Native gal-8 exists as a monomer and its two CRDs are structurally different with

different specificities for sugar residues which does not require complex N-Glycans for binding (Patnaik et al., 2006)

Galectin-9 is a 40 kDa protein consisting of 353 amino acids. The sequence identity between the N- and C-terminal CRDs is 35% (Nagae et al., 2006; Cao et al., 2007; Nagae et al., 2008). Galectin 9 is a lactose binding protein reported to be expressed in a subpopuation of cells present in peripheral blood leukocytes, lymphoid tissues (Sahin et al., 1995; Tureci et al., 1997; Nagae et al., 2008), kidney, intestine and thymus (Wada et al., 1997) controlling wide range of activities like T-cell development (Wada and Kanwar, 1997; Wada et al., 1997), chemo-attraction of eosinophils, induction of apoptosis and down-regulating of effector TH_1 responses (Matsushita, 2000; Kageshita et al., 2002; Zhu et al., 2005; Nagae et al., 2008). Galectin-10 has been originally isolated as Charcot-Leyden crystal because of its unusual solubility properties and spontaneously crystallization in eosinophil mediated inflammatory tissues (Charcot and Robin, 1853; Leyden, 1872; Kilpatrick, 2002). It constitutes >7% of the total protein content of a typical eosinophils (Ackerman et al., 1993) and displays weak lactose and mannose binding specificity in soluble and crystalline state, respectively (Swaminathan et al., 1999; Kilpatrick, 2002).

Very little information is available on gal-11 and 13, except that they contain one carbohydrate recognition domain (Kilpatrick, 2002). Galectin-11 (also called GRIFIN for "galectin related interfiber protein") was found to be present in the lens of the eye, suggesting a role for this protein in the maintenance of immune privilege in this vulnerable tissue (Rabinovich et al., 2002; Rabinovich et al., 2007).

Galectin-12, a recently identified member of this family, contains two CRDs and has shown growth inhibitory properties (Yang et al., 2001; Hotta, 2001). Expression of galectin-12 is high in peripheral blood leukocytes and adipocytes, but very low or undetectable in many tissue and cell lines, except in those of myeloid origin with the potential to undergo terminal differentiation (Yang and Liu, 2003).

Gal-14 is a novel sheep galectin specifically expressed in eosinophils, but this galectin is similar in sequence to the first galectin domain of galectin-9. It has been shown to have galactoside-binding activity and has been named galectin-14 (Dunphy et al., 2000; Dunphy et al., 2002). Galectin-14 seems to be released into the airway lumen in response to allergen challenge; therefore a possible role in immune defense (Dunphy et al., 2002) is suggested.

Galectin-15, also known as OVGAL11 and a previously uncharacterized member of the galectin family of secreted β-galactoside lectins containing a conserved carbohydrate recognition domain and a separate putative integrin binding domain, was discovered in the uterus of sheep (Gray et al., 2004; Gray et al., 2005). It is concentrated near and on the apical surface of the endometrial luminal epithelia and localized within discrete cytoplasmic crystalline structures of conceptus trophectoderm (Tr) (Gray et al., 2004; Rabinovich et al., 2007). Galectin-15 protein is functional in binding lactose and mannose sugars and immunologically identical to the unnamed *Mr* 14,000 (14 kDa) protein from the ovine uterus that forms crystalline inclusion bodies in endometrial epithelia and conceptus Tr (Gray et al., 2004).

Structural Classes of β-galactoside binding lectins

Based on protein architecture and its ability to function as a cross linker, the galectin family has been divided into three subgroups as shown in Fig. 2 (Vasta et al., 2004).

Prototype

The proto-type galectins (galectin-1, 2, 5, 7, 10, 11, 13, 15 and 14) consist of a single CRD per subunit with a short N-terminal sequence and a single core protein domain (Houzelstein et al., 2004; Rabinovich et al., 2007; Nagae et al., 2008). Most of these galectins are homodimers or multimers as in the case of sponge galectins (Miarons and Fresno, 2000), but some others, such as mammalian galectin-5, -7 and -10 (Kopitz et al., 2003) exists in monomeric form. They also show distinct tissue specific and developmentally regulated expression in a variety of different sources (Cooper, 2002). Dimerization of prototype galectins involves self-association of monomer subunits at sides opposite their CRDs through non-covalent interactions to create bivalent lectins (Brewer et al., 2002; Rabinovich et al., 2007). Thus, the CRD pockets and any bound ligands face away from each other, an arrangement unlikely to allow multivalent binding to closely clustered ligands (Brewer, 2002; Brewer, 2004; Dam et al., 2005). However, these dimers can very effectively crosslink separated ligands, even forming chains or crystalline networks of the divalent lectin bound to di- or multivalent ligands (Sacchettini, 2001; Liu and Rabinovich, 2005).

Tandem-repeat

These galectins (Galectin-4,-6,-8,-9 and 12) are composed of two non identical core galectin domains joined either directly or via a linker peptide of variable length (Toscano et al., 2007). Their CRDs are positioned in such a way that they can simultaneously bind to multivalent ligands, greatly enhancing the binding avidity

(Arata et al.,1997; Hirabayashi et al., 2002) as opposed to homo-functional cross-linking by dimeric prototype galectins (Cooper, 2002).

Chimeric type (Galectin-3)

These galectin types are composed of a protein domain joined to a distinct N-or-C-terminal domain (Rabinovich et al., 2007) which is responsible for interactions between subunits, thus facilitating its oligomerization (Liu and Rabinovich, 2005). In vertebrates, the only chimeric galectin yet discovered is galectin-3, but several other chimeric galectins are apparent in invertebrates (Dumic et al., 2006). Galectin-3 is composed of an N-terminal domain with several repeats of a peptide sequence rich in proline, glycine and tyrosine residues followed by a C-terminal galectin domain (Dumic et al., 2006). The intact C-terminal galectin domain remains with its lectin activity intact, but loses much of its propensity to multimerize (Kuklinski and Probstmeir, 1998). Galectin-3 monomers are in equilibrium with higher order oligomers in solution and galectin-3 precipitates as a pentamer with multivalent oligosaccharides (Ahmed et al., 2004). This lectin binds to multi-glycosylated proteins with positive co-operativity, suggesting that galectin-3 monomers, after ligand binding, recruit additional lectin molecules to form a complex of multivalent interactions (Brewer et al., 2002; Ahmed et al., 2004). The biologic functions attributed to galectin-3 are thus likely to depend upon both ligand crosslinking and oligomerization (Nieminen et al., 2005; Stillman et al., 2005; Chen et al., 2006; Toscano et al., 2007; Nieminen et al., 2007).

Secretion and biosynthesis of β-galactoside binding lectins

Although galectins are often reported to be present on cell surfaces or in extracellular matrix, they lack recognizable secretion signal sequences and do not pass through the standard Endoplasmic reticulum/Golgi pathway (Leffler, 2001; Cooper, 2002), with the possible exception of a sponge galectin (Miarons and Fresno, 2000). Instead, most galectins have characteristics typical of cytoplasmic proteins, such as an acetylated N-terminus, free sulfhydryls and lack of glycosylation. Nevertheless, there is strong experimental evidence that at least some galectins are, indeed, secreted, albeit by novel non-classical mechanisms (Hughes, 1999; Boulianne et al., 2000; Leffler et al., 2004). Galectin secretion from cells is tightly controlled during development and several unrelated factors, including cytokines as well as adhesive and membrane fusion can modulate secretion (Hughes, 1999). Pulse chase experiments also demonstrated that the lectin is actively secreted from cells, rather than leaked from

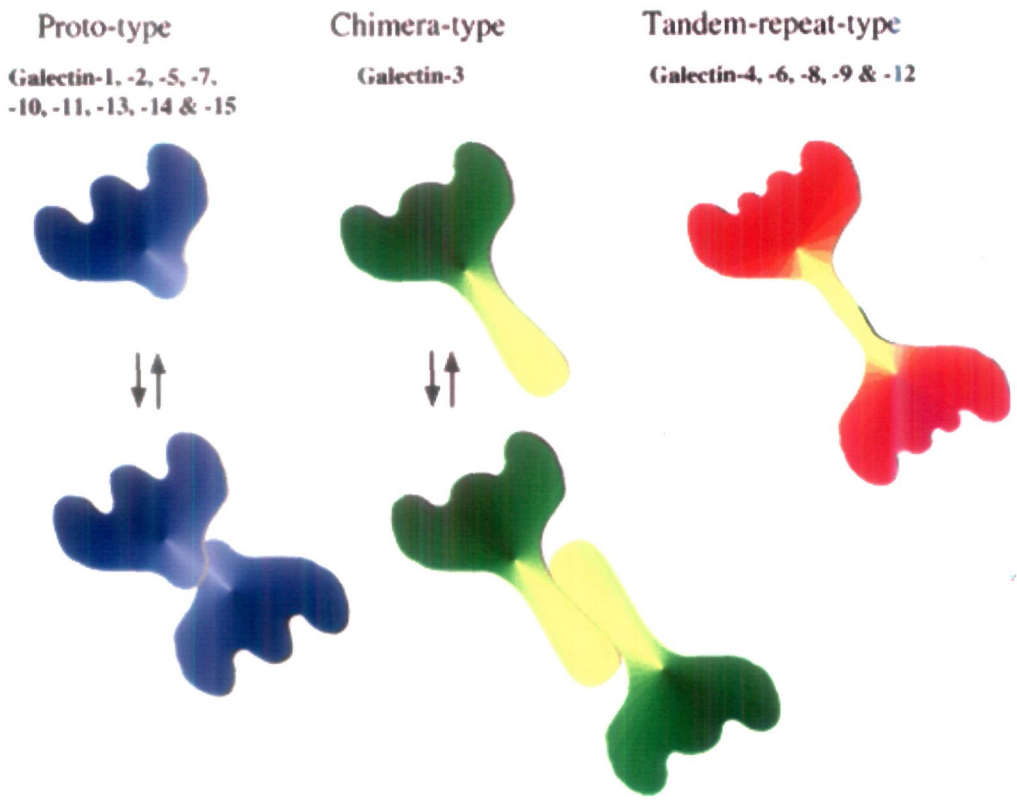


Figure 2. Pictorial representation of three types of lectins.

The first group consists of proto-type galectins with one carbohydrate binding domain or carbohydrate recognition domain (CRD). The second group consists of chimera-type galectins that have one CRD and another non-carbohydrate-binding domain linked together. The third group consists of tandem-repeat-type galectins with two CRDs. Although proto-type and chimera-type galectins have only one CRD, two molecules bind to each other (dimerization) so that they can actually bind to two carbohydrate chains as tandem-repeat-type galectins do (some proto-type galectins are unlikely to form a dimer) (Stillman et al., 2005).

damaged cells (Cooper, 2002; Hughes, 2001). Upon secretion, galectins typically bind to and stay associated with glycoproteins and glycolipids on the cell surface, or within the surrounding extracellular matrix to complete its folding and stability (Stillman et al., 2005). Thus, while galectins are abundant in tissues where they are made, however, only low levels are found in serum and serum levels of galectins do not reflect the rates of synthesis in tissues (Iurisci et al., 2000; He and Baum, 2004).

Purification strategies of β-galactoside binding lectins (Galectins)

In general, soluble β-galactoside binding lectins have been isolated by affinity chromatography on asialofetuin or lactosyl-Sepharose 4 B columns (Ola et al., 2001; Ola et al., 2007). First, the fresh animal tissue is solubilized with phosphate buffer saline containing reducing agents and competing sugars in order to dissociate soluble β-galactoside binding lectin from insoluble substances of starting tissues (Kasai and Hirabayashi, 1996). The acetone powder prepared from animal tissue has been rarely used to extract lectin (Den and Malinzak, 1977). Then soluble β-galactoside binding lectin is isolated from crude extract by ammonium sulphate fractionation followed by affinity chromatography. The bound protein is eluted with lactose. The lectin activity is measured by hemagglutination assay using fresh rabbit and human erythrocytes treated with trypsin or neurominidase (Muramoto et al., 1999; Ola et al., 2001). In addition, multiple forms of lectin have also been isolated from human and rat lung by employing ion exchange chromatography on DEAE-cellulose or sephadex column followed by chromatography on CM-sephadex column (Cerra et al., 1985; Sparrow et al., 1987) in the presence of protease inhibitors to minimize the proteolytic degradation of lectin.

Physicochemical properties of β-galactoside binding lectins (Galectins)

These lectins are soluble proteins bearing an affinity for β-galactoside containing moieties, besides possessing a carbohydrate recognition domain with conserved sequence elements that require a reducing environment for action but no divalent ions (Hasan et al., 2007). They are distinguishable from all other lectins by their low molecular weights, ranging from 14-36 kDa, dimeric nature and their variable sub-cellular location (Rabinovich et al., 2007). By virtue of their multivalency, galectins are able to cross link cell surface glycoconjugates and iniatiates cell biological response (Rabinovich et al., 2007). These lectins binds to N-acetyllactosamine with relatively low affinity (dissociation constant (K_d) in the range of 90-100 μM), but they bind to glycoproteins containing polylactosamine

sequences with a high affinity (K_d-1μM) (Brewer et al., 2002; Dam et al., 2005). While CRDs of all galectins share affinity for N-acetyllactosamine found on many cellular glycoproteins, individual galectins can also recognize different modifications of this sugar, demonstrating the fine specificity of certain galectins for tissue or developmentally specific ligands (Brewer, 2004; Ahmed et al., 2004).

In the isolectric focusing experiment, 90 % of rat lung lectin moved with the isoelectric point (pI) of pH 5.5. However, a faint band with pI of pH 5.5 was also observed (Clerch et al., 1988). Amino acid composition of galectins obtained from different sources of several mammals is found to be similar, if not identical (Shahwan et al., 2004). The total number of acidic amino acid residues is found to be significantly higher than the total number of basic amino acid residues. All lectins studied so far have blocked N-termini and an acetyl group is demonstrated to be the blocking group (Leffler et al., 2004; Rabinovich et al., 2007). Galectins occasionally contain cysteine residues, but no disulfide bond is formed and all SH groups are in a free state. Till date, no galectins are glycosylated with an exception of sponge galectin (Miarons and Fresno, 2000). Cloning of cDNAs revealed that galectins are synthesized without a signal sequence (Kasai and Hirabayashi, 1999), strongly suggesting that galectins are designed as intracellular proteins (Leffler et al., 2004).

Ligands for Galectins

Despite the large number of β-galactoside-containing glycoconjugates present in the cellular milieu, few glycoproteins from cell extracts bind to particular galectins in-vitro (Barondes et al., 1999) suggesting that these may be the interactions that are physiologically significant. Among naturally occurring glycoconjugates, glycoproteins that contain polylactosamines are especially good ligands for galectins (Liu, 2000; Rabinovich et al., 2007). Of these, laminin, a glycoprotein with many polylactosamine chains, has been implicated as a natural ligand for galectin- 1 (Hughes, 2001) and is also bound by galectin-3 (Liu, 2000). Galectin- 1 has also been shown to bind to other glycoconjugates including polylactosamine-rich lysosome-associated membrane proteins (Imbe, 2003) that are sometimes found on the cell surface, a lactosamine-containing glycolipid on olfactory neurons (St John and Key, 1999; Horie and Kadoya, 2004) and integrin $\alpha_7\beta_1$ on skeletal muscle cells (Hadari et al., 2000).

Carbohydrate binding domain and specificity

Galectins have in common a highly conserved carbohydrate binding site (CRD) with affinity for lactose and N-acetyllactosamine (Fig. 3) (Barondes et al., 1999; Vasta et al., 2004; Ahmed and Vasta, 2008), formed by part of the six stranded antiparallel β-pleated sheets that form an extended sandwich with a typical jellyroll topology and is around 130 amino acid long (Liao et al., 1994). The core sequence of this domain lies between the 30^{th} and the 90^{th} residues and is encoded by a single exon (Cooper and Barondes, 1999). The number and arrangement of the CRDs may vary and has been used as a basis of their classification. The galectin CRD has a concave side forming a groove long enough to hold a linear oligosaccharide made of up to four monosaccharide units (Loris, 2002; Leffler et al., 2004). The fine specificity of the two CRDs may differ (Hirabayashi et al., 2002; Ideo et al., 2003; Zick et al., 2004) displaying tightest interaction with galactose residue, but interaction with glucose is also significant, making the afiinity for lactose 50-150 folds higher compared to galactose for most galectins (Toscano et al., 2007). However the carbohydrate binding cleft can accommodate an additional one to three sachharide residues suggesting the reason for polylactosaminoglycans as good ligands for galectins (Cho and Cummings, 1995; Stowell et al., 2004; Sorme et al., 2004). In fact polylactosaminoglycans are bound by the galectins more tightly than lactose/N-acetyllactosamine due to the presence of secondary sites for interaction with more extended oligosachharides (Leppanen et al., 2005) in addition to the primary site, which accommodates lactose/N-acetyllactosamine (Toscano et al., 2007). Each galectin has a unique specificity, for example the affinity of galectin-1 for blood group A tetrasaccharide is about 100 fold lower than that of galectin-3 and certain complex mucin derived saccharide that bind galectin-3 well, do not bind galectin-1 at all (Brewer, 2002).

Galectin-1, -3 and -5 also differs in their affinity for certain disachharides such as Gal-β-1-β GalNAc (Brewer, 2002). The two chicken galectins, C14 and C16, were shown to differ in their fine specificity for a panel of synthetic lactose derivative (Barboni et al.,2000).

Mutivalency of β-galactoside binding lectins

Most galectins are divalent, either by self-association or by including two CRDs in one protein (Gabius et al., 2002). All galectins identified till date can be structurally classified into three basic types (Vasta et al., 2004), which achieve di- or higher valency by distinct mechanisms. Multimerization is a common feature across various

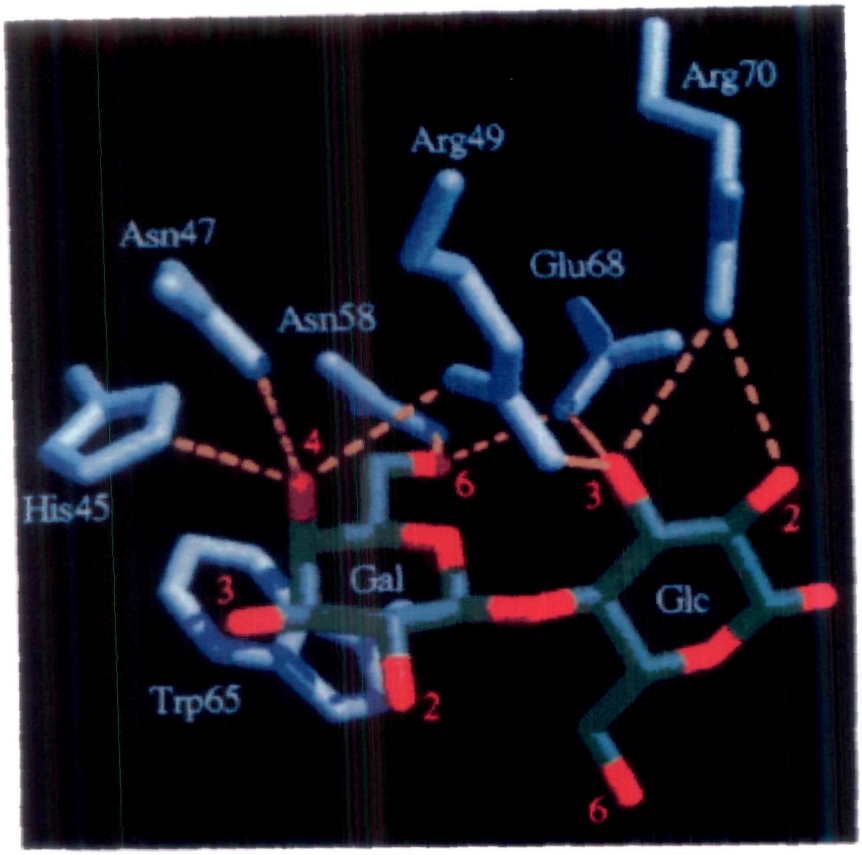


Figure 3. Fine structure of interaction between galectin-2 and lactose.

The sugar residues are shown in green with red oxygen atoms and position numbers. The amino acid side chains interacting with the saccharides are shown in light blue. The principal hydrogen bonds between amino acid side chains and the sugar residues are shown as yellow dotted lines (Barondes et al., 1999).

families of carbohydrate binding proteins, perhaps because binding interactions of individual CRDs with even their most favored carbohydrate ligands are of relatively low affinity and multimerization can greatly increase binding avidity for multivalent or clustered ligands (Rabinovich et al., 2007). However, in studies of a range of di- or multivalent galectins, only some show enhanced avidity for multivalent ligands (Hirabayashi et al., 2002). This may mean that for some galectins, the significance of di- or multivalency is to provide them the ability to crosslink ligands (Cooper, 2002). Indeed, there is considerable evidence that some galectins regulate association of their glycoconjugate ligands on cell surfaces, in extracellular matrices, or both (Brewer, 2002; Rabinovich et al., 2007). Monovalent galectins might function to competitively block such cross-linking activity (Cooper, 2002).

Crystal structure of β-galactoside binding lectin

The refined X- ray model of S- lectin consists of 133 amino acid residues in one monomer and 132 amino acid residues in the other monomer, two N-acetyllactosamine molecules and 154 water molecules (Liao et al., 1994). The S-lectin dimer forms a 22-strand anti-parallel β-sandwich, with the N and C termini of each monomer at the dimer interface (Fig. 4). The two molecules are related to each other by a non-crystallographic 2-fold rotation perpendicular to the β-sheets displaying a jelly roll topology containing a β-hairpin insertion with the first and second parallel β-strands omitted (Liao et al.,1994). The structure reveals that there is one carbohydrate-binding site per monomer, which is located on the same side of the β -sandwich and on the far ends of the dimer 46 Å apart. The integrity of the dimer is maintained by the β-sheet interactions across the monomers and by the formation of a hydrophobic core common to both. In S-lectin the two β-sheets of the monomers extend continuously across the dimer interface and all direct protein carbohydrate interactions involve side chains located on β-strands (Liao et al., 1994).

Carbohydrate Binding-Site Interactions

The well-defined electron density of both carbohydrate molecules clearly indicates the α-anomeric form for the GlcNAc unit (Liao et al., 1994). Most amino acid residues involved in sugar binding are invariant in all sequences of S-lectins. The binding-site depression is shaped to complement the galactose moiety with extensive Van der Waals contacts and a network of electrostatic interactions (Fig. 5). In addition, a water molecule mediates the interactions of the side chain of Asn-46 with the 3-OH and 4-OH of galactose. The aromatic side chain of the conserved Trp-68 stacks adjacent to

Figure 4. Fold of S-lectin: Highlighting secondary structure motifs of the dimmer.

β strands are shown as ribbons and the N-acetyllactosamine molecules are shown as yellow stick models. The model was generated by the computer program RASTER 3 D written by David Bacon (University of Alberta, Canada) (Barondes et al.,1994).

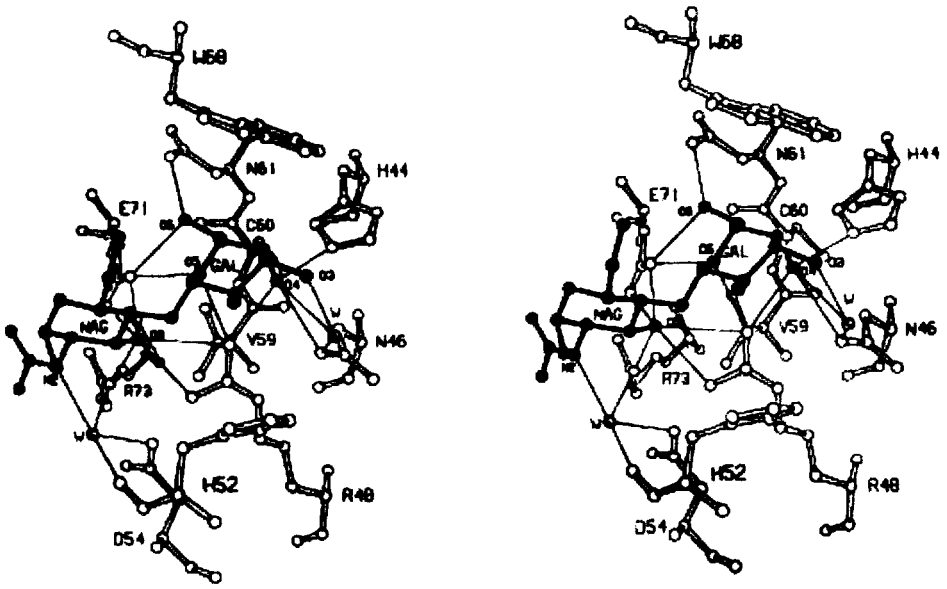


Figure 5. Stereoscopic representation of S-lectin carbohydrate binding site.

Bonds between carbohydrate atoms are solid and those between protein atoms are open. The electrostatic interactions between protein and sugar atoms are shown in thin N46 lines. For clarity, protein-protein electrostatic interactions of residues involved in sugar binding are not indicated; these include the salt bridges between Arg-48 and Asp-54, Arg-73 and Asp-54 and Arg-73 and Glu-7 (Liao et al., 1994).

the galactose ring. Such Van der Waals interactions between sugar and aromatic side chains are quite common in protein-carbohydrate complexes (Vyas, 1991). The axial 4-OH of galactose is the main determinant of the S-lectin specificity which forms two key electrostatic interactions i.e one with the N" atom of Arg-48 and the other with the N^{e} atom of His-44. The interactions of the GlcNAc moiety with the protein are less extensive than those of H 44 of galactose, whereas the binding of N-acetyllactosamine to S-lectin is 5-fold tighter than the binding of lactose (Ahmed et al., 1990; Vasta et al., 2004) which may be attributed to Van der Waals interactions between the N-acetyl group and the side chains of Arg-73 and Glu-71. The carbohydrate-protein interactions are supported by exquisite protein-protein electrostatic interactions, assuring optimal side-chain conformations (Fig. 5). Most striking is the spatial disposition of the charged residues Arg-48, Asp-54, Arg-73 and Glu-71, which together form a network of three salt bridges unprecedented in other sugar-binding proteins of known structure. Of these, Asn-46, Glu-71 and Arg-73 have been previously suggested as essential for binding by site-directed mutagenesis (Hirabayashi and Kasai, 1991). These structural data explain why those amino acid residues that are directly and indirectly associated with the carbohydrate binding are conserved.

Environment of Thiol Groups.

The crucial role that the oxidation states of the S-lectin thiol groups play in regulating function in vivo is still a mystery. According to the refined structure (Liao et al., 1994), the thiol groups of Cys-42 and Cys-60 are reduced and buried whereas Cys-2 thiol group is disordered and Cys-16, Cys-88 and Cys-130 sulphydryl moieties are oxidized and solvated. No thiol group is directly involved in sugar binding and all thiol groups bind heavy atoms without impairing sugar binding. Cys-60 is the only cysteine residue close to the binding site. Its main-chain atoms are involved in the formation of the active site depression and its side chain is buried and surrounded by hydrophobic residues (Liao et al., 1994). Site-directed mutagenesis studies of the 14-kDa S-lectins, replacing Cys-60 and Cys-2 by serine residues, did not inhibit sugar binding, consistent with the structural information (Abbot and Feizi, 1991; Hiarabayashi and Kasai, 1991).

Hemolytic and membrane perturbing action of β-galactoside binding protein

Animal lectins have been of great interest recently, as their various important functions have been suggested. One of the most probable roles of β-galactoside

binding lectins is to act as humoral factors in the defense mechanism against various pathogenic agents. Moreover, gal-1 preferentially binds to ganglioside GM_1 on neuroblastoma cells to exert growth control (Kopitz et al., 2001; Seibert et al., 2003; Kopitz et al., 2003) and that it harbors a site to interact with hydrophobic tails of oncogenic H-Ras (Rotblat et al., 2004). The underlying mechanism to carry out this role is its glycan binding property present on the cell membranes, thereby causing lysis of the cells. Lytic action of some lectins have been ascribed to enzymatic activity (Hittelet et al., 2002), perturbation of the activities of membrane-associated enzymes (Lowe and Marth, 2003), or pore formation in the membranes (Yu et al., 2002).The lectin dependent association of glycans may affect membrane features such as fluidity, permeability and osmofragility (Gupta et al., 2006). In addition, lectin might also interact with hydrophobic membrane patches adding to its impact on membrane characteristics (Gupta et al., 2006).

Functions of β-galactoside binding lectins

Lectins bearing specificity towards galactoside residues are involved in wide array of functional activities owing to its potential of recognition and discrimination between potential glyco-substrates. Some of their principle roles are discussed below:

Role of β-galactoside lectins in inflammatory and immunomodulatory responses

Among the various functions of galectins, the role in the modulation of the immune response has been very well documented. Recent research indicated that gal-1 ameliorates phospholipase A2 induced edema (Rabinovich et al., 2000; Rabinovich et al., 2002; Rabinovich and Gruppi, 2005), regulates the release of soluble mediators from lipopolysaccharide stimulated macrophages, blocks neutrophil extravasation (Elola et al., 2005), mast cell degranulation (Rubenstein et al., 2004) and nitric oxide synthesis (Rabinovich et al., 2004). Moreover, presence of Gal-1 in bone marrow derived mesenchymal (stromal) cells suggests its implications in bone marrow derived cell differentiation and mobilization (Panepucci et al., 2004; Kadri et al., 2005; Kiss et al., 2007). In addition, gal-1, displays pleiotropic immunomodulatory functions, including regulation of lymphocyte survival and cytokine secretion in autoimmune system, transplant diseases, parasitic and viral infection models (Santucci et al., 2000; Santucci et al., 2003; Baum et al., 2003, Rabinovich et al., 2004; Levroney et al., 2005).

Role of β-galactoside lectin in cell-cell and cell matrix interactions

Galectin plays a pivotal role in adhesion and migration of cell mediated by cross-linking a mosaic of extracellular matrix glycoproteins like laminin, fibronectin, lysosome associated membrane proteins and CD45 (Wada and Makino, 2001; Rabinovich et al., 2004). Despite specific binding of galectins to these glycoconjugates, its anti or pro-adhesive role still remains obscure. For example, gal-1 promotes the adhesion of ovarian carcinoma cells to extracellular matrix (Rabinovich et al., 2004), whereas it inhibits the adhesion of myoblast to laminin by blocking the laminin receptor integrin α7β1 from recognizing laminin (Matarrese et al., 2000a). Similarly, gal-3 preferably mediates adhesion of the neutrophils to laminin in comparison to melanoma (Sato et al., 2002). While gal-3 contributes to cell interactions between dendritic cells and naive T lymphocytes in lymph nodes (Rabinovich et al., 2002, Ilarregui et al., 2005), a recent study revealed that galectin-3 even disrupts thymocyte interactions within the thymic microenvironment, thus acting as a de-adhesion molecule (Villa et al., 2002).

Role of β-galactoside lectin in pre-mRNA splicing

The process of deletion and reconstitution of splicing activity, assayed in cell free system, suggested that galectins are major factors involved in pre-mRNA processing (Park et al., 2001; Liu et al., 2002). Other techniques such as co-localisation studies (Vyakarnam et al., 1998; Wang et al., 2007) and immunofluorescence microscopy (Liu et al.,2002) also revealed nuclear speckled structures containing galectin and known splicing factors, thus providing additional evidence to confirm the role of galectins in mRNA splicing. The domain structure for gal-1 and gal-3 suggested that homologous CRD was necessary and sufficient for splicing activity (Park et al.,2001; Liu et al., 2002), which thereby interacts with various functional proteins constituting the spliceosome assembly (Pellizzoni et al., 1998; Park et al., 2001).

Role of β-galactoside lectin in cell growth and apoptosis

Several early studies provided correlative evidence for an association between galectin expression and cell proliferation. Galectin-3 is expressed at high levels in a wide range of neoplasms, including spontaneous, viral, ultraviolet and chemically induced tumors (Plazk et al., 2004; Dumic et al., 2006) suggesting the possibility that the galectins may have a role in regulation of cell growth (Liu et al., 2002; Dumic et al., 2006). Some pro-apototic galectins, such as gal-1 and gal-9, directly initiate death by cross-linking cell surface receptors, whereas intracellular expression of other

galectins, such as gal-7, potentiates other death signals (Rabinovich et al., 2004). Galectins affect cell fate decisions in a variety of tissues and cell types. For example, gal-1 kills T cells, B cells and prostate and breast cancer cell lines, suggesting that gal-1 may recognize a common carbohydrate ligand on diverse cell surface receptors to initiate a common intracellular death pathway (Hernandez and Baum, 2002; Stillman et al., 2005). Multiple galectins have also been shown to influence viability in a single cell type. Complex expression patterns of galectins and their carbohydrate ligands may allow temporal regulation of cell viability during development, tissue remodeling and inflammation (Hernandez and Baum, 2002; Leffler et al., 2004). In addition, some galectins can act extracellularly to induce apoptosis in concert with other stimuli. For example, addition of exogenous gal-8 induced apoptosis of serum-starved carcinoma cells in a carbohydrate dependent manner (Hadari et al., 2000). Furthermore, galectin-1 also induces apoptosis of breast and prostate cancer cell lines (Yang and Liu, 2003) and melanoma cells (Stillman et al., 2005).

Role of β-galactoside lectins in regulation of cell cycle

Factors playing significant role in cell growth and apoptosis simultaneously function in controlling cell cycle, thus suggesting that galectins also play significant role in regulating cell cycle. For example, galectin-1 has also been shown to inhibit growth of mouse embryonic fibroblast at relatively low concentrations (Wells and Mallucci, 1991; Rabinovich et al., 2001) and induce cell cycle arrest during the S-to G2 transition of mammary cell lines (Wells et al., 1999; Novelli et al., 1999; Yang and Liu, 2003). It also inhibits the IL-2 induced proliferation of phytohemagglutinin activated T lymphocytes, IL-independent proliferation of T lymphoma cells, concavavalin A (Con A)-stimulated rat T cells (Rabinovich et al., 2004), human neuroblastoma cells (Rubinstein et al., 2004; Rabinovich and Gruppi, 2005), human leukemia T cells (Nakahara et al., 2005) and murine fibroblasts (Nakahara et al., 2005).

Role of β-galactoside lectins in cancer and metastasis

The myriad critical roles played by galectins ranging from cell signaling to apoptosis make them potent tumirogenic molecules. Galectins are often overexpressed in cancerous cells and cancer-associated stromal cells (Danguy et al., 2002; Lahm et al., 2004; van den Brüle et al., 2004; Takenaya et al., 2004). In general, this altered expression correlates with the aggressiveness of the tumors and the acquisition of metastatic phenotype, indicating that galectins might modulate tumor progression and

influence disease outcome (Danguy et al., 2002; Greco et al., 2004). There is increasing evidence that galectins have important functions in several aspects of cancer biology (Rabinovich et al., 2005; Liu and Rabinovich, 2005), including tumor transformation (Paz et al., 2001), apoptosis (Matarrese et al., 2000b; Rabinovich et al., 2002; Matarrese et al., 2005) and cell growth regulation (Yamaoka et al., 2000; Kopitz et al., 2001). In addition, galectins are also involved in various steps of tumor metastasis, including tumor cell adhesion (Levy et al., 2001; van den Brüle et al., 2003), homotypic cell aggregation (Tinari et al., 2001; Glinsky et al., 2003), invasiveness (Hittelet et al., 2002), angiogenesis (Nangia-Makker et al., 2000). Gal-3 could be used as a prognostic marker for thyroid cancer, colon cancer and cancers of pancreas, bladders, head and neck squamous epithelial cells, stomach and kidneys (Plzak et al., 2004; van der Brule et al.,2004, Hasan et al., 2007). Similarly, galectin-1 is also involved in cancer development as it anchors the molecule Ras, which is involved in cellular transformation (Paz et al., 2001). Increased malignant potential of human thyroid tumors (Xu et al., 1995), glioma (Rorive et al., 2001) and prostate adenocarcinoma (Ellerhorst et al., 1999; van der Brule et al., 2001; Rabinovich et al., 2004) has been correlated with enhanced expression of galectin-1 both on the surface of the tumor cells as well as the stromal tissue surrounding the tumors (van der Brule et al., 2001; He and Baum, 2004; Stillman et al., 2005).

Therapeutic applications of galectins

The study of galectins has been an area of active research in biomedical sciences as these molecules play important roles in a variety of biological processes (Hasan et al., 2007). Research over the past decade indicated that the local administration of galectin-1 prevented clinical and histopathological manifestations of many well known diseases such as Mysthania Gravis and experimental encephalomyelitis, (Santucci et al. 2000; Wada and Makino, 2001; Rabinovich et al., 2004; Rabinovich and Gruppi, 2005), autoimmnune encephalitis (Kilpatrick, 2002; Rabinovich and Gruppi, 2005), collagen induced arthritis (Rabinovich et al., 1999; Wada and Makino, 2001), colitis (Santucci et al., 2000), hepatitis (Santucci et al., 2003), autoimmune uveitis (Toscano et al., 2007) and anti-glomerular basement membrane (GBM) glomerulonephritis (Tsuchiyama et al., 2000). In anti-GBM nephritis in experimental rats, the administration of gaelctin-9 induced apoptosis of activated CD 8 positive cells and ameliorated proteinuria and renal tissue injuries (Tsuchiyama et al., 2000). The ability of galectin-1 to suppress the allogeneic T-cell response through apoptotic

and non-apoptotic mechanisms (Rabinovich et al., 2002; Stillman et al., 2005) suggests its potential use for immunosuppression in organ transplantation and graft versus host diseases (Toscano et al., 2007; Rabinovich and Gruppi, 2005). For example, galectin-1 treatment significantly improved reconstitution of normal splenic architecture following hematopoietic stem-cell transplantation and, similar to its effects on autoimmune settings, this β-galactoside-binding protein reduced the production of T1-type cytokines (Baum et al., 2003; Toscano et al., 2007).

Possible usage of galectins in the therapy of atherosclerosis and diabetic vascular complications by modulating macrophage function and advanced end glycation products clearance is suggested by several studies (Nachtigal et al., 1998; Wada and Makino, 2001; Stillman et al., 2005). Galectin-7 is also reported to increase the chemotherapeutic efficacy of cisplatin in treatment of urothelial cancer through the accumulation of intracellular reactive oxygen species (Matsui et al., 2007).

Furthermore, galectin display antimicrobial activity by acting as immunosuppressant during various infections caused by *T. Cruzi,* (Zuniga et al., 2001) *Leismania major* (Pelletier and Sato, 2002; Pelletier et al., 2003), HIV (Lanteri et al., 2003) and Nipah Virus (Levroney et al., 2005).

Interestingly, the discovery of anti-galectin antibodies in pathological sera and autoimmune disorders like Chagas' cardiomiopathy (Giordanengo et al., 2001), non-endemic loiasis (Rabinovich et al., 2002; Rabinovich et al., 2004) and Hodgins's disease (Kilpatrick 2002; Rabinovich and Gruppi, 2005) opens new diagnostic and potentially therapeutic avenues for researchers in application of galectins as suitable target for novel drugs and pharmacological molecules.

Detection of altered galectin expression also serves as excellent diagnostic markers in various carcinomas (Hasan et al., 2007; Stillman et al., 2005) and inflammatory cells (Rabinovich et al., 2005; Rabinovich and Gruppi, 2007).

Direct administration of oxidized galectin has implication in axonal regeneration and functional recovery after peripheral nerve injury (Kadoya et al., 2005). Galectin especially gal-1 has innumerable direct therapeutic application in mammalian nervous system which has been discussed in detail in the next section.

Presence and role of β-galactoside binding lectin in mammalian nervous system

Localization

β-galactoside binding lectin are ubiquitously distributed throughout the mammalian tissue including skin (Kuwabara et al., 2002; Rabinovich et al., 2004), muscle

(Rubenstein et al., 2004), lymph node (Stillman et al., 2005), thymus (Kuwabara et al., 2002) , lung, spleen and placenta (Rabinovich et al., 2004). They are present on the cell surface and the extracellular matrix as well as in the cytoplasm and the nucleus of the cells comprising these tissues (Imbe et al., 2003). In the mammalian nervous system, β-galactoside binding lectins are highly expressed (Perillo et al., 1998; Rabinovich, 1999; Stillman et al., 2005) particularly localized in neurons in the central nervous system (CNS) and peripheral nervous system (PNS) (Pesheva et al., 2000; Yang et al., 2006; Mok et al., 2007) in developing animals, but its distribution is restricted to the peripheral nervous tissue in adults (Hynes et al., 1990; Perillo et al., 1998; Horie et al., 1999; Imbe et al., 2003). In human brain, galectin-3 is expressed by fibroblasts, macrophages, activated microglial cells, a subpopulation of dorsal root ganglions (DRG) and Schwann cells following nerve injury (Woo et al., 1990; Reichert et al., 1994; Stillman et al., 2005). Galectin-1 expression has not been well characterized in human brain; however, in animal models, galectin-1 is expressed in a subpopulation of DRG, primary sensory neurons, olfactory and motor neurons (Horie and Kadoya, 2004) as well as astrocytes, perivacular cells and microvessels (Sango et al., 2004; Stillman et al., 2005; Mok et al., 2007). Galectin-1 mRNA persists in dorsal root ganglia neurons at later development stages and is maintained in adult sensory neurons (Horie and Kadoya, 2004). The mRNA is also expressed in motoneurons in the spinal cord and brain stem (Hynes et al., 1990). Immunohistochemical study for gal-1 in normal rats also showed immunoreactivity in DRG neurons and their axons as well as in the spinal cord, where motoneurons and their axons were specifically positive (Horie et al., 1999; Kadoya et al., 2005;). Galectin-1 immunoreactivity was observed in regions containing Schwann cells and regenerating axons (Horie et al., 1999). In the olfactory system, galectin-1 is expressed by ensheathing cells both in olfactory bulb of the embryonic and adult rat. In the adult rat, galectin-1 was preferentially expressed by olfactory ensheathing cells in the nerve fiber layer of the ventromedial and lateral surfaces of the olfactory bulb (John and Key, 1999; Mok et al., 2007).

In the central nervous tissue of adult mammal, galectin-1 seems to be expressed in the restricted sites like retinal pigment epithelium, outer limiting membrane and outer plexiform layer in bovine and rat retinas (Uhera et al., 2001). In the adult rat brain, galectin-1mRNA is expressed in pineal gland and in neurons in nuclei of cranial nerves, red nucleus, locus ceruleus and cerebeller nucleus (Akazawa et al., 2004). The

expression level of the mRNA is up-regulated in motoneurons in facial nucleus after transfection of facial nerve (Akazawa et al., 2004; Kadoya et al., 2005).

Functions

Despite the high expression of galectins in mammalian brain, mystery regarding the functional activities of these proteins largely remains unfolded. However, galectin-1 knockout mice demonstrate defects in olfactory neuron targeting (Puche et al., 1996; Kadoya et al., 2005), thus indicating its potential functions in nervous system. It is a well known fact that galectin-1 is a homodimer with a subunit molecular mass of 14.5 kDa and exhibits β-galactoside binding activity only in the reduced form. But surprisingly the axonal regeneration activity of this lectin is promoted by its oxidized form (Kadoya et al., 2005) containing three intermolecular disulfide bond (Cys^{2}-Cys^{130}, Cys^{16}-Cys^{88}, Cys^{42}-Cys^{60}) (Inagaki et al., 2000). This has been proved by several experiments including purification of galectin-1 from oxidative environments and subjecting to axonal regeneration activity in a dose dependent manner in the DRG explant model and fluorogold tracer technique where recombinant oxidized galectin was administered locally to acellular autografts (Fukaya et al., 2003; Horie and Kadoya, 2004; Kadoya et al., 2005). The disulfide bond formation alters the structure of galectin-1 as to confer the novel ability to promote axonal regeneration (Kadoya et al., 2005). In contrast, reduced form of lectin did not promote axonal regeneration but induced marked hemagglutination (Inagaki et al., 2000). Thus it can be said that oxidized form of galectin-1 is not a member of gaelctin family, because it lacks β-galactoside binding ability and therefore it is more appropriate to term it as oxidised galectin in order to distinguish it from galectin-1 having lectin activity. In addition to axonal regeneration, oxidized gal-1 also advances the restoration of nerve function after peripheral nerve injury (Hori and Kadoya, 2004; Kadoya et al., 2005). The administration of recombinant oxidized galectin to the nerve injury site was found to increase both the number and diameter of regenerating myelinated fibers, especially of medium sized fibers (Kadoya et al., 2005). The mechanism behind the promotion of peripheral nerve regeneration is still obscure. But recent reports suggest that macrophages are the target cells and that the oxidized galectin-1 stimulates macrophages to secrete a factor that promotes axonal growth and Schwann cell migration (Horie et al., 2000). This essential function of oxidized gaelctin-1 for peripheral nerve regeneration is thought to be specifically different from other neurotrophic factors. Thus, it can be said that oxidized gaelctin-1 is potentially

therapeutic for functional restoration after peripheral nerve injury. In contrast, a study by Kopitz et al., (2004), suggested that galectin-1, acting as a lectin promotes axonal regeneration of hippocampal axons in vitro. The fact that hippocampal neurons are components of the central nervous system may be basic to explain this different behavior of galectin-1, considering the general physiological differences between peripheral and central nervous systems and, in particular, their distinct capacities for regeneration (Kopitz et al., 2004).

The endogenous galectin-1 may also contribute to the establishment of neuropathic pain after the peripheral nerve injury by increasing some sensory excitatory neuropeptide receptor into the spinal cord which is responsible to the development of mechanical hypersensitivity in neuropathic pain (Imbe et al., 2003; Horie and Kadoya, 2004).

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by loss of motor neurons in the cerebral motor cortex, brainstem and spinal cord (Rowland and Schneider, 2001). Oxidized form of galectin-1 is reported to promote the survival of degenerating motor neurons in ALS (Hong et al., 2005), thus suggesting a potential therapeutic effect of galectin-1 for patients with ALS. Furthermore, galectin-3, which is over-expressed in scrapie-infected brain tissue (Reimer et al., 2004; Mok et al., 2006), is reported to play important role in chronic degeneration during prion infection of CNS (Mok et al., 2007), probably by interacting with the lysosomal membrane proteins which contribute to the lysosomal autophagy for degrading organelles and long lived proteins during scrapie infections (Mok et al., 2007). Thus, a detrimental role of galectin-3 is suggested in prion infection of central nervous system.

Moreover, galectins play vital roles in the development and the potential treatment of brain tumors by directly regulating the balance between cell survival and cell death, modulating cell motlity and impacting signaling through Ras pathway (Hughes, 2001; Paz et al., 2001; Hernandez and Baum, 2002; Stillman et al., 2005). In addition, by regulating immune responses, galectins may also contribute the efficacy in brain tumor patients to a variety of experimental immuno and vaccine based chemotherapy (Stillman et al., 2005). Increased expression of galectin-1 mRNA and immunoreactive galectin-1 is reported in astrocytic tumors ranging from low grade astrocytoma to malignant glioma (Yamaoka et al., 2000; Camby et al., 2002; Stillman et al., 2005), thereby promoting tumor cell invasion and dissemination in in-vivo (Rorive et al.,

2001). During tumor metastasis galectin-1 is suggested to increase cell motility associated with reorganization of the actin cytoskeleton and increased expression of RhoA, a protein that modulates actin polymerization and depolymerization (Stillman et al., 2005). It is also suggested that they promote detachment of tumor cells from the initial tumor site and facilitate migration into the surrounding brain parenchyma (Camby et al., 2002; Stillman et al., 2005). Galectin-3 is a useful diagnostic marker to distinguish subtypes of astrocytic and glial tumors (Neder et al., 2004) and may also have extracellular roles in brain cancer progression (Stillman et al., 2005). For example, due to defective tumor cell glycosylation, galectin-3 do not adhere to the glioma cell lines, thereby leading to decreased glioma cell adhesion at the primary tumor site which may contribute to gliomas cell invasion and migration (Kuklinski et al., 2000; Debray et al., 2004). Thus, the significance of galectins in brain tumors and its invasion is largely contributed to its ability to bind to neural cells and neural glycoconjugates, resulting in aggregation and formation of neurite bundles.

As it is evident from the review of literature, the β-galactoside specific lectins are an important class of proteins expressed in mammalian tissues which carry out various important functional roles. These proteins isolated from different animals expressed in various tissues may differ significantly in structural and physiochemical properties. The studies on brain lectins carried out so far are primarily confined to bovine, rat, mouse and human brains. The possible occurrence of the lectins in other mammalian brains, their structure and functional relationship are yet to be studied. Keeping this in mind the present study was undertaken to investigate the β-galactoside specific lectins from buffalo brain. The purpose of this study was not only to look for lectins which display specific sugar specificity but to start a comprehensive study which will contribute to the pool of basic information necessary for the understanding of their biological functions.

OBJECTIVES OF THE PRESENT WORK

1. Soluble β-galactoside binding lectins are an important group of proteins, which have assumed significance in recent years in view of their various roles in all spheres of mammalian nervous system. Thus, the present work was carried out to explore the possible occurrence of lectin in brain tissue from water buffalo and to study its functional and structural properties. Water buffalo was selected for this study as it is an easily available and an inexpensive source of mammalian lectin.
2. Soluble β-galactoside binding lectin was purified by ammonium sulphate fractionation followed by gel filtration chromatography. A complete characterization of its properties such as molecular weight, stokes radius, carbohydrate specificity, temperature and pH optima etc were carried out.
3. Since we obtained glycosylated form of soluble β-galactoside binding lectin, an effort was made to understand the role of glycosylation in first ever glycosylated brain lectin reported. Therefore, we have made an attempt to study the comparative stability of glycosylated and deglycosylated form of lectin against various denaturing agents.
4. In addition, oxidized form of lectin has been reported to have several implications in mammalian nervous system. Therefore, we evaluated the effect of H_2O_2 on buffalo brain lectin in terms of its functional and structural parameters using UV, fluorescence and circular diochroism spectroscopy techniques.
5. Lectins, irrespective of their source, owing to their glycan binding nature disrupt the organization and structure of biomembranes leading to lysis of the cells. Thus, an effort has also been undertaken to study the cytolytic nature of purified lectin using erythrocytes as a model.
6. A change in the glycosylation pattern of erythrocytes membrane takes place during the onset and progression of various carcinomas. Therefore, we examined the appearance and disappearance of β-galactoside moieties on red blood cells from breast and prostate cancer patients using buffalo brain lectin as a diagnostic tool.

Materials and Methods

MATERIALS

Chemicals and reagents used in the present study were obtained from the following sources as detailed below. Glass distilled water was used in all experiments.

Genei Pvt. Ltd., Bangalore, India

Molecular weight markers (medium range).

Hi-Media, India

Agar agar, agarose, Freund's complete adjuvant, Freund's incomplete adjuvant, sodium azide.

Pharmacia Fine Chemicals, Sweden

Blue Dextran.

Qualigens Fine Chemicals, India

Acetone, ammonium sulphate, ammonium persulphate, cupric sulphate, cupric chloride, ethyl alcohol, glacial acetic acid, hydrochloric acid, isopropanol, methanol, monosodium dihydrogen orthophosphate, di-sodium hydrogenorthophosphate, sodium carbonate, sodium chloride, sodium hydroxide, sodium lauryl sulfate, sodium potassium tartarate, sulphuric acid, thiourea, tris hydroxyl methyl amino acid methane, hydrogen peroxide, ethylene glycol, sodium periodate.

Sigma Chemicals Co., USA

Sephadex G_{50-80}, sephadex G_{100}, coomassie brilliant blue R-250, iodoacetate, iodoacetamide, ovalbumin.

Sisco Research Laboratories (SRL), India

Acrylamide, N'N methylene bis-acrylamide, bovine serum albumin, comassie brilliant blue R-250, EDTA, Folin-ciocalteau'phenol reagent, glycine, phenol, N'N-dithiobisnitro benzoic acid, cysteine, cytochrome C, D-galactosamine, galactose, methyl β-D-galactoside, trypsin, glutraldehyde, soyabean trypsin inhibitor.

Fresh buffalo brain tissues were routinely collected from the slaughter house (within 30 minutes after the animals were killed) in ice and processed for lectin purification within 2 hours of collection. Albino rabbits were used for collection of erythrocytes and raising of antibodies.

METHODS

Isolation of β-galactoside binding lectin from buffalo brain

Brain homogenization

The freshly preleved brain was rinsed in normal saline solution. The meningeal membranes were carefully removed. Total brain was cut into small pieces and suspended in 75 mM sodium phosphate buffer saline pH 7.2, containing 0.15 M NaCl, 5 mM β-ME, 150 mM lactose, 0.02 % sodium azide and 10 mM EDTA in the ratio of 1 gm of brain tissue/2 ml of buffer. Homogenization was carried out at 4°C in a stainless steel vessel using mixer table homogenizer at full speed for three periods of 1 min with 1 min. intervals. The homogenate was further centrifuged in a Beckman J 2-21 cooling centrifuge (JA-20 rotor, 10000 rpm for 30 min) at 4° C and the supernatant was collected.

Fractionation of brain homogenate

The supernatant of homogenate obtained from above steps was re-centrifuged at 40,000 rpm for 1 hour in Beckman L 8-60 M ultracentrifuge using Ti rotor. The supernatant was collected and kept on ice. To this soluble protein solution an equivalent weight of solid ammonium sulphate was slowly added to obtain a final 40 % saturation and kept at 4°C for 3 hours. The precipitated proteins were removed by centrifugation at 10,000 rpm for 30 min. in a cooling centrifuge and the supernatant was further brought to 70% saturation with solid ammonium sulphate. After agitation for 3 hours at 4° C the precipitate was collected and dissolved in minimal amount of 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl (PBS), 0.02 % sodium azide, 30 mM lactose and 5 mM β-ME. After extensive dialysis against the same buffer, the sample was loaded on a sephadex $G_{50\text{-}80}$ column.

Gel filtration chromatography

Sephadex $G_{50\text{-}80}$ column was prepared as recommended by Peterson and Sober (1962) at room temperature. The gel was allowed to swell in sufficient amount of distilled water for 6 hours in boiling water bath. The gel fines were removed by suspending the gel in 75 mM PBS, pH 7.2 and allowing 90-95% of the gel to settle down. The remaining gel in supernatant was rapidly removed by suction. A previously cleaned glass column mounted on a sturdy vertical support was filled to one third of its length with the distilled water in order to check leaks and flush air bubbles from the dead space. The deareated gel slurry was then gently poured with

the help of a glass rod into the column with care to avoid generation of air bubbles. The column was left standing overnight. Flow rate was increased gradually and after accomplishing a constant flow rate higher than that required for final elution the column was adjusted to the required flow rate. The packed column was thoroughly washed with two bed volumes of 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl and 5 mM β-ME (operating buffer). In order to check the uniform packing and to determine the void volume of the column, 0.2 % (w/v) solution of blue dextran in operating buffer was passed through the column. The volume of blue dextran or protein solution applied on the column was not more than 2-3% of the total bed volume.

The column was thoroughly washed and equilibrated with 75 mM PBS, pH 7.2 containing 5 mM β-ME and 30 mM lactose prior to application of the sample. The dialysed sample obtained after 40%-70% ammonium sulphate fractionation was applied at room temperature to the chromatographic column. Two ml fractions were collected and assayed for protein concentration and hemagglutinating activity. The fractions of protein peak were again dialyzed against 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl and 5 mM β-ME to remove lactose and tested for hemagglutination activity. Samples showing maximum activity were pooled and concentrated for study. The protein content was quantitated by Folin's phenol reagent by the method of Lowry et al. (1951). Homogeneity of the preparation was analysed by applying the sample on 10 % polyacrylamide gel electrophoresis (PAGE).

Colorimetric analysis

Determination of protein concentration

Protein was estimated by the method of Lowry et al. (1951). Aliquots of protein solution were taken in a set of tubes and final volume was made up to 1 ml with 75 mM sodium phosphate buffer (pH 7.2). Five ml of alkaline copper reagent (containing one part of 1 % (w/v) copper sulphate and 2 % (w/v) sodium potassium tartarate in 1% (w/v) sodium hydroxide and sodium carbonate) was added and then after 10 min of incubation at room temperature, 0.5 ml of 6 N Folin-Ciocalteau's phenol reagent was added. The tubes were instantly vortexed. The colour developed was read at 660 nm after 30 min against a reagent blank. A calibration curve was prepared using BSA standard.

Determination of carbohydrate content

The procedure described by Dubois et al. (1956) was followed. Two ml of sugar solution containing 10 to 100 μg of carbohydrate was pipetted in test tubes and 0.5 ml of 80% phenol was added to it. This was followed by the addition of 5.0 ml concentrated sulphuric acid, the stream of acid being directed against the liquid surface rather than the side of the test tube in order to obtain good mixing. The tubes were vortexed and placed for 20 min. at room temperature to cool. The colour was stable for several hours. The absorbance of the characteristic yellow orange colour was measured at 490 nm for quantitation of hexose content against blank prepared by substituting buffer for sugar solution.

Determination of sulphydryl groups

The method used was essentially that described by Ellman (1959). For the determination of exposed -SH groups, appropriate amount of protein was added in 100 mM Tris-EDTA buffer, pH 8.0 to give a total volume of 3.0 ml of the solution. 0.1 ml DTNB solution (40 mg DTNB in 10 ml of 0.1 M Tris HCl, pH 8.0) was added and the colour developed was read at 412 nm after 15 min. A reagent blank was used to account for the absorption of the reagent at 412 nm. Cysteine (0.125 mM) was taken to obtain standard plot of SH groups. Thiol groups in μ moles were calculated using the standard plot of cysteine per μ mole of protein.

Hemagglutination assay

Hemagglutination activity was routinely assayed with rabbit erythrocytes by the methods of Lis and Sharon (1972) as described below.

Preparation of RBC suspension

A. Untreated erythrocytes

A blood sample from a healthy rabbit was obtained by vein puncture in 3% trisodium citrate solution as an anticoagulant used in a ratio of 4:1.The erythrocytes were then centrifuged at room temperature in a clinical table top centrifuge (2000 rpm for 5 min) and washed three to four times with 75 mM PBS, pH 7.2 (5 ml per 1 ml packed cells). Washed erythrocytes were made 4 % (v/v); (3x 10^8 cells /ml) by adding 75 mM PBS, pH 7.2.

B. Enzymatically modified erythrocytes: (Trypsinized erythrocytes)

An 8 % (v/v) suspension of washed erythrocytes was prepared in 75 mM PBS, pH 7.2. Trypsin was added to a final concentration of 100 μg /ml (0.1 ml of trypsin

solution to 1 ml of erythrocytes suspension) and incubated for 1 hour at 37°C. The trypsinized erythrocytes were washed four to five times with 75 mM PBS, pH 7.2 and 4% (v/v) suspension was prepared for further use.

Hemagglutination test

A 50 µl of 75 mM PBS, pH 7.2 was added to each row of wells on a microtitre 'V'plate, starting with the second well. To each of the first two wells 50 µl of the solution to be tested for activity was added and mixed by shaking the plate by hand. 50 µl of a liquid from the second well was transferred to the adjacent one, mixed and transferred 50 µl from the latter well to the next one and so on up to the last well. This protocol produced two fold serial dilutions of the tested material i.e. 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, etc. 50 µl of the 4% (v/v) erythrocyte suspension was added to each well, mixed and left for 1 hour at room temperature. Highest dilution giving agglutination was noted. A mat of erythrocytes covering the well showed the occurrence of agglutination. In absence of agglutination, erythrocytes appeared as a button at the tip of the well. One agglutinating unit was arbitrarily described as the minimal of material required to cause full agglutination under the above conditions. The titre of the tested solution was expressed as a reciprocal of the highest dilution showing agglutination.

Determination of carbohydrate specificity by inhibition of hemagglutination

Qualitative determination

Two mM solutions of the sugars to be tested were prepared. The titre of the lectin preparation was determined and diluted such that 50 µl had its titre 4. In wells of a microtitre plate, 50 µl of the test carbohydrate solutions were mixed with 4 agglutinating units of the lectin and kept for 5-10 min and 50 µl of a 4% suspension of erythrocytes were added. The degree of agglutination was determined in different wells after 1 hour. Absence of agglutination indicated that the lectin was specific for the sugar in the well.

Quantitative determination

Two fold serially diluted test sugar solutions were incubated for 5-10 min at room temperature with 4 agglutinating units of lectin in microtitre plates. Agglutination was observed after 1 hour of addition of 50 ul of 4% erythrocyte suspension to each well.

The highest dilution of the test sugar giving complete inhibition of hemagglutination was noted.

Slab gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed by Tris-Glycine system of Laemmli (1970) using slab gel apparatus manufactured by Biotech, India. Routinely 10% and 12.5% acrylamide gels were used. Concentrated stock solution of 30% acrylamide containing 0.8% bisacrylamide and 1.0 M Tris (pH 6.8 and 8.8) were prepared and mixed in appropriate amount to give the required final concentration. The solution was deareated and poured in the mould formed between the two glass plate (8.5 x 10 cm) separated by 1.5 mm thick spacers. Bubbles and leaks were avoided. A comb providing a template for 7 wells was inserted into the gel before the polymerization began. After polymerization the comb was removed and wells were overlaid with the running buffer. Protein samples were prepared to give final concentration of 0.0625 M in Tris HCl (pH 6.8), 10 % glycerol and trace of bromophenol blue as a tracking dye. Electrophoresis was performed in electrode buffer containing 0.025 M Tris, 0.2 M glycine at 100 V till the tracking dye reached the bottom of the gel.

SDS-Gel Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was essentially performed by the method of Laemmli (1970). Stock solutions of 30% acrylamide containing 0.8% bisacrylmide, 1.0 M tris HCl (pH 6.8) and 10% of SDS were mixed in specific proportion to give desired percentage of acrylamide. Protein samples were prepared to give a final concentration of 1% SDS (w/v), 0.5 % β-ME (v/v), 0.25 M tris HCl, pH 6.8 and 10 % glycerol (v/v) and a trace of bromophenol blue as a tracking dye. Samples were then heated in a boiling water bath for about 3-5 min; electrophoresis was carried out at 100 V for approximately 3 hours in electrode buffer containing 0.025 M Tris, 0.2 M glycine and 0.02 % SDS.

Coomassie blue staining

After electrophoresis the gels were stained with five gel volumes of 0.25% coomassie brilliant blue R-250 in 5 % methanol and 10 % acetic acid for at least 4 hours. For destaining, the gels were incubated in 5 % methanol and 7.5 % acetic acid at room temperature with vigorous shaking.

Molecular weight determinations

The molecular weight of the brain lectin was determined under the native conditions using gel filtration and under denaturing condition by SDS-PAGE.

Gel filtration

The molecular weight of the native buffalo brain lectin was computed from its elution volume in sephadex G_{100} column which was prepared as discussed before using the method of Peterson and Sober (1962). The column was calibrated by determining the elution volumes of several globular proteins of known molecular weights like Bovine serum albumin (68 kDa), ovalbumin (45 kDa), soyabean trypsin inhibitor (20.1 kDa) and cytochrome c (12.4 kDa). Purified buffalo brain lectin was applied to the column and 3 ml fractions were eluted with 75 mM PBS, pH 7.2 containing 30 mM lactose, at a flow rate of 15ml/hr. The data were analysed according to the theoretical treatment of Andrews (1964). The linear plot between V_e/V_o and log M was used for calculating the molecular weight of the lectin.

SDS-PAGE

The subunit molecular weight of BBL was calculated by the procedure of Weber and Osborn (1969) using its mobility on SDS-PAGE. The mobility of marker proteins determined under identical conditions was plotted against the logarithm of molecular weight. The analysis of data indicated a linear relationship between log M and relative mobility (Rm). The plot between log M and relative mobility was used for calculating molecular weight of the lectin.

Determination of stokes radius

The stokes radius of buffalo brain lectin was determined by the methods of Laurent and Killander (1964) using gel filtration data. The proteins bovine serum albumin (35.5Å), ovalbumin (27.3 Å), soyabean trypsin inhibitor (22.6 Å) and cytochrome C (16.4 Å) were taken as standards. The value of Kav of each marker protein was calculated from the formula Kav=Ve-Vo/Vt-Vo by substituting the values of Ve (elution volume), Vo (void volume) and Vt (total volume of column). A graph of the square root of the negative logarithm of Kav versus stokes radius gave a linear plot which was used for calculating stokes radius of the brain lectin.

Thermal stability

The temperature stability of the native and deglycosylated lectin (125µg/ml) was determined by incubating 100 µl samples in 75 mM PBS pH 7.2 containing 5 mM

β-ME at various temperature (30°C to 70°C) for 30 min, cooling it on wet ice and titrating with trypsinized rabbit erythrocytes using microtitre plate assay.

pH stability

To determine the optimal pH and stability of native and deglycosylated lectin activity, the proteins (125μg/ml) in 50 μl of normal saline containing 5 mM β-ME were incubated with 50 μl of following buffers, 0.1 M sodium acetate buffer (pH 6.5-7.5), 0.1 M tris HCl buffer (pH 8.5-9.5) and 0.1 M glycine-NaOH buffer (pH 10.5-11.5) for 24 hours at 4°C. The hemagglutination activity of the lectin was then assayed using trypsinized rabbit erythrocytes on microtitre plates.

Chemical modification of active groups of lectin

The rate of the reactions with 70 mM alkylating agents like iodoacetate and iodoacetamide in 75 mM PBS pH 7.2 containing 1 mm β-ME and with oxidizing agents (5 mM H_2O_2 in the absence of β-ME) in the same buffer were determined at room temperature. After designated times the modified brain lectin was titrated for hemagglutination assay.

Effect of detergents, denaturants and metal ions on lectin induced hemagglutination

The effect of denaturants on native and deglycosylated lectin (125μg/ml) was determined by incubating protein with urea, guanidine HCl and thiourea in the concentration range of 0.5 M to 8.0 M in 75 mM PBS, pH 7.2 containing 5 mM β-ME. Titre value of each sample was determined using microplate assay. Native BBL sample in 75 mM PBS, pH 7.2 containing 5 mM β-ME served as control (initial activity).

The effect of different detergents was also monitored by incubating native and deglycosylated proteins (125μg/ml) with SDS (0.1-2 mg/ml), Triton-X (0.5-5 % (v/v)), and Tween 20 (0.5-5 % (v/v)) in 75 mM PBS, pH 7.2 containing 5 mM β-ME. Titre value of each sample was determined using microplate assay. Native BBL sample in 75 mM PBS, pH 7.2 containing 5 mM β-ME served as control (initial activity).

To examine the effect of divalent cations on lectin activity, demetallization of purified lectin was carried out using 0.1 M EDTA followed by remetallization of the sample with 0.1 M $CaCl_2$ and $MnCl_2$, $[Sr(CH_3COO)_2]$, $MgCl_2$, $NiCl_2$ in 75 mM PBS, pH 7.2 containing 5 mM β-ME. Hemagglutination activity of each sample was tested by microtitre plate assay using rabbit RBCs. Native BBL sample in 75 mM PBS, pH 7.2 containing 5 mM β-ME served as control (initial activity).

Equilibrium dialysis

The binding of lactose to BBL was quantitatively studied in 75mM PBS, pH 7.2 containing 5 mM β-ME by equilibrium dialysis in a dialysis bag (3.0 ml capacity) made from Sigma cellulose membrane (inflated diameter 6 mm, width 10 mm). The dialysis bag containing 1ml (100 μM) of the lectin solution was placed in plastic vials containing 1.0 ml of lactose solution in the range of 40 μM-400 μM. After capping the vials, the dialysis bag was mechanically shaken for 24 hours at 37°C. After attainment of equilibrium, the decrease in lactose concentration was estimated in the dialysate, outside the dialysis bag by the method of Dubois et al., (1956). The amount of lactose bound per mole of lectin was calculated according to Scatchard analysis. It should be noted that no detectable binding of lactose by the dialysis bag was observed.

BBL readily reacted with the specific carbohydrate, according to the following reaction.

$$L + nS \rightarrow LSn \qquad (1)$$

$$K_{ass} \rightarrow \frac{[LSn]}{[L][S]n} \qquad (2)$$

$$\upsilon \rightarrow n K_{ass}[S]/1 + K_{ass}[S] \qquad (3)$$

where n is the number of carbohydrate binding sites in the lectin dimer and K_{ass} is the association constant for the interaction of S (or lactose) with L (or lectin). Equation 3 can be arranged to Eq. 4.

$$\upsilon/[S] \rightarrow -K_{ass}\upsilon + nK_{ass} \qquad (4)$$

The amount of lactose bound per mole of lectin i.e., υ, was determined in triplicate and plotted according to Eq. 4.

Brain cell aggregation assay

The brain cell aggregation activity was determined using dissociated cells from goat and buffalo brain without any chemical treatment. One-gram tissue was isolated from the telencephalon of each brain and mechanically dissolved into separate cells by standardized method using a dissociation chamber containing a magnetic stirrer (Bologna et al., 1982, Caron et al., 1987). Perfectly isolated cells were automatically and reproducibly obtained without adding any chemical substance. The cell suspension was fixed in 4% (v/v) glutraldehyde in 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl. After one hour, the cells were washed in 75 mM PBS, pH 7.2, rinsed twice in 0.2 M glycine, and dissociated again. Isolated cells were finally

suspended (6.25×10^5 cells/ml) in 75 mM PBS, pH 7.2. 100 µl of purified lectin (10µg/ml) was added to tubes containing 100 µl of a suspension of isolated fixed cells. After rotation at 70 rpm for 60 min, small aliquots were carefully removed and the number of particles (single cells and clumps) per unit volume was determined using a Maleuzz hematocytometer. The rate of agglutination was expressed as the % decrease in particle number (Caron et al., 1987). Similarly, the effect of temperature and pH on brain cell aggregation by BBL was also measured by incubating BBL and isolated cells at different temperatures (20-80°C) and buffers of various pH values (3.5-11.5) and counting the number of cells as discussed above.

Preparation of periodate oxidized Buffalo Brain Lectin

Deglycosylation of lectin was carried out according to the method of Rasheedi, et al. (2003). Protein sample (1mg/ml)in 75 mM PBS pH 7.2 was prepared and treated with 10 mM sodium periodate solution in a molar ratio of 5:1. Reaction mixture was incubated for 15 min at room temperature in dark. The oxidation process was stopped by adding 0.5 ml ethylene glycol per ml of sample. The quenched sample was then dialysed at room temperature overnight against 75 mM sodium phosphate buffer pH 7.2 containing 0.15 M NaCl and 5 mM β-ME.

Spectroscopy

UV spectroscopy

The UV spectra of native lectin in 75 mM PBS containing 5 mM β-ME and oxidized lectin by adding 5 mM H_2O_2 (in the absence of β-ME) were measured on a Beckman UV 640 spectrophotometer in the wavelength region 220-350 nm.

Fluorescence spectroscopy

The intrinsic fluorescence of the lectin was measured at 25±0.2° C in Hitachi-F 2000 spectrofluorometer (Tokyo, Japan). The protein was excited (λ ex) at 280 nm. Corrected emission spectra were recorded with excitation and emission band widths of 10 nm. Appropriate control containing substances used for the treatment were run and correction made wherever necessary.

The change in intrinsic fluorescence of native lectin in PBS (45µg/ml) containing 5 mM β-ME and oxidized lectin by adding 5 mM H_2O_2 (in the absence of β-ME), in presence as well as in absence of 0.1 M lactose was followed by measuring the emission fluorescence in the wavelength region of 300-400 nm. Similarly, effects of

various denaturants (0.5-8 M of GdnHCl, urea and thiourea) on the fluorescence spectra of native and deglycosylated lectin were also measured.

Circular diochroism spectroscopy

Circular diochroism of native lectin and after various treatments were measured in a Jasco J-810 spectropolarimeter equipped with a temperature controlled sample cell holder. Spectra were recorded with scan speed of 100nm/min and with a response time of 1 sec and 1 nm band width. Each spectrum was the average of four scans. Measurements in the far UV (200-250 nm) as well as in the near UV (250-350 nm) regions were taken using 250μg/ml and 1 mg/ml of BBL, respectively.

Fourier Transform Infrared Spectroscopy

Infrared spectroscopy was done to see the changes in the secondary structure components (conformational changes) in BBL in the presence of oxidizing agents. The spectra were truncated between 1740 and 1520 cm^{-1} and baseline recorded. The equipment used was NICOLET (ESP) 560 spectrophotometer (USA) equipped with a transmission OMNIC ESP 5.1 software and a DTGS detector. BBL solutions (0.15 mg/ml) were prepared in sodium phosphate buffer (10 mM, pH 7.2).

Original spectra of native and treated BBL at 37°C were recorded with a resolution of 4 cm^{-1} and 128 scans. The changes in peak frequency and intensity were then assigned to conformational change within the protein. Native BBL was taken as control.

Immunological techniques

Immunization

Antibodies against BBL were raised in healthy adult male albino rabbits (New Zealand strain) weighing 1.5-2.0 kgs. Prior to immunization the rabbits were bled for obtaining serum that served as control in the studies. They received a primary immunization with 300 μg of purified buffalo brain lectin emulsified in equal volume of complete Freund's adjuvant through a subcutaneous injection at several sites on the back. Weekly secondary immunization of 150 μg of protein in Freund's incomplete adjuvant was administered after 2 weeks. A similar second and third booster was given in the third and fourth weeks, respectively and the animal was bled after an additional one week. The blood collected was allowed to coagulate at 20°C for 3 hrs, the anti-sera was decomplemented at 37°C for 30 min, collected in small aliquots and kept frozen at -20^0C until further used.

Ouchterlony immunodiffusion

Immunodiffusion was performed essentially according to the method of Ouchterlony (1962). One percent molten agarose prepared in normal saline containing 0.1% sodium azide and 30 mM lactose (to prevent the interaction of lectin with agarose or serum glycoproteins), was poured on clean petriplate and allowed to solidify at room temperature. Required number of wells were cut from agarose and stored at 4°C. Antisera 10 to 40 µl and required amount of antigen were added in the central and peripheral wells, respectively. The petriplate was incubated at 37 °C for 4 hours and then at 4°C overnight in order to get a clear precipitin band. The petriplate was photographed under proper illumination. The cross reactivity of antibodies was also checked against sheep and goat brain lectin.

Direct binding ELISA

Elisa was performed essentially as described by Aotsuka et al., (1979). Polystyrene (96 wells) microtitre plates coated with 100 µl of antigen at a concentration of 2.5 µg/ml in TBS (10 mM Tris, 150 mM NaCl, pH 7.4), kept for 2 hrs at room temperature and overnight at 4° C. The antigen coated wells were washed three times with TBS-T (20 mM Tris, 144 mM NaCl, 2.68 mM KCl, pH 7.4 with 500 µl Tween-20) to remove unbound antigen. Unoccupied sites were blocked with 150 µl of 1.5 % BSA in TBS for 3 hrs at room temperature. After washing the plates with TBS-T, the serially diluted antiserum (100 µl/well) were added to each well and plates were incubated for 2 hrs at room temperature. The bound antibodies were assayed by an appropriate anti-rabbit goat IgG labeled with horse radish peroxidase using hydrogen peroxide and tetramethylbenzidine as substrate. The plates were incubated for 1 hr and absorbance was read at 450 nm. Each sample was coated in duplicate. Results were expressed as a mean of A (test)- A (control).

Competition ELISA

The antigenic specificity of the induced antibodies was determined by competition inhibition experiments (Hasan et al., 1991). Varying amount of inhibitors (0-20 µg/ml) were mixed with a constant amount of antiserum (1:100 dilution), and the mixture was incubated at room temperature for 2 hrs and overnight at 4°C. The immune complex thus formed, was coated in the microtitre wells (instead of serum). The remaining steps were same as that of direct binding ELISA. The results thus obtained were expressed as % inhibition.

Percent inhibition= 1-A (*inhibited*)/A (*uninhibited*) × *100*

A (inhibited) = absorbance at 450 nm in presence of varying amounts of inhibitors.

A (uninhibited) = absorbance at 450 nm in the absence of inhibitors.

Dot blot analysis

The specificity and the immunological cross reactivity of buffalo brain lectin antisera with other galactoside binding lectins present in liver, lung and heart was demonstrated by standard dot blot assay taking respective tissue homogenates. Two microlitres of the purified lectin sample was spotted on nitrocellulose paper and the blot was blocked by overnight incubation with 5% BSA solution. The blot was first incubated with rabbit anti-lectin antisera (1:50) and then with peroxidase coated goat anti-rabbit antibodies at the dilution of 1:1000 for 2 hrs at 37° C in a moist chamber. Extensive washings of the blot were carried out with 0.05 % Tween-PBS at every step to remove excess reagents. The binding of the BBL and of the tissue lectins with anti-buffalo brain lectin antibodies were visualized by developing the blot with a mixture of 0.1 % H_2O_2 containing 1 mg/ml diaminobenzidine

Interaction of BBL with erythrocyte membrane

Preparation of erythrocyte suspension

A blood sample from a healthy rabbit (adult, male, New Zealand white strain) was obtained by vein puncture in 3 % trisodium citrate solution as an anticoagulant used in a ratio of 4:1. The erythrocytes were then centrifuged 2000 rpm for 5 min, plasma and buffy coat were discarded and pelleted cells were washed three times with 75 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Finally, a 2 % erythrocyte suspension was made (2 ml of packed erythrocytes in 100 ml PBS). Trypsin was added to a final concentration of 100 µg /ml (0.1 ml of trypsin solution to 1ml of erythrocytes suspension) and incubated for 1 hour at 37°C. The trypsinized rabbit RBCs were washed four to five times with 75 mM PBS, pH 7.4 and finally 10% suspension was prepared.

Determination of osmofragility of lectin agglutinated erythrocytes

For the determination of osmofragility of erythrocytes (Raghuramulu et al., 1983); from the cell suspension prepared as above, aliquots of 200µl were taken in different tubes. To these aliquoted cell suspensions, 100 µl of either lectin in 75 mM PBS, pH 7.4 were added and the mixture was incubated for a period of 1 h at room temperature (37°C) for the agglutination to take place. After this 1.5 ml of hypotonic solution of varying concentrations (i.e. 0.35-0.85% NaCl) was added to the above reaction. The

cells were then incubated for a period of 4 h at room temperature with intermittent gentle mixing. At the end of the incubation period, the tubes were centrifuged at 2000 rpm for 5 min and the supernatant was collected. Extent of hemolysis was measured by taking absorbance at 540 nm. Results were reported as percent lysis where lysis of similar volume of RBC with the same amount of distilled water was taken as 100%.

Effect of various colloids on the osmofragility of lectin agglutinated erythrocytes.

For protection of osmotic lysis, the lectin agglutinated erythrocytes were incubated with 1.5 ml of 75 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4 with 30 mM of ribose, galactose, mannose or lactose. The cells were incubated at room temperature for 6 h with gentle mixing. Hemolysis was subsequently quantitated as described above. Erythrocytes without lectin, incubated at similar conditions, served as control.

Effect of lectin on superoxide induced damage of erythrocytes membrane

Red blood cell suspensions (200 µl) were taken in different test tubes. To these aliquoted cell suspensions 100 µl of lectin (100µg/ml) was added and the mixtures were incubated for one hour at room temperature. After this, the agglutinated cells were exposed to superoxide radical (O_2^-), generated from a pyrogallol auto-oxidation system by adding 10 µl of pyrogallol solution (0.02 M freshly made in H_2O_2) and incubated for a period of 20 min at room temperature, and the erythrocytes were recovered after centrifugation of the sample at 3000 rpm for 5 min. The cells were washed thrice with PBS and the supernatant was collected for analyzing the hemoglobin released. The released oxyhemoglobin concentration in supernatants was measured by the equation of Winterbourn (1990) using absorbance measurements at 560, 577 and 630 nm, with the following equation:

$[\text{Oxy Hb}] = 119\ A_{577} - 39\ A_{630} - 89\ A_{560}$.

The susceptibility of erythrocytes membranes to hypochlorous acid induced damage in the presence and absence of lectin

Red blood cell suspensions (200 µl) was mixed with 100 µl (100µg/ml) in 75 mM PBS, pH 7.4 containing 5 mM β-ME and incubated for 1 hour for agglutination to take place. The agglutinated cells were treated with different concentrations of hypochlorous (HOCl) acid at 22° C for 20 min. Then, the cells were washed three times with excess of cold PBS and re-suspended in PBS as 10% suspension. HOCl was added as a single bolus of 25 mM l^{-1} stock solution of sodium hypochlorite (NaOCl) in PBS buffer to RBC suspension and mixed by vortexing. At pH 7.4 this

solution contains a mixture of HOCl and OCl^- at approximately 1:1 ratio and is subsequently referred to as HOCl (Vissers et al., 1998). The concentrations of OCl^{-1} were determined spectro-photometrically using an absorbance coefficient of 350 M^{-1} l cm^{-1} at 292 nm and pH 9.0 (Morris, 1996). The susceptibility of erythrocytes to HOCl induced oxidative damage was measured in terms % hemolysis. The process of hemolysis of agglutinated erythrocytes treated with HOCl was monitored by hemoglobin release as discussed above.

Preparation of human erythrocytes suspensions of cancer patients

Both pre and post-operative venous blood was collected from breast and prostate cancer patients in tubes containing EDTA. Within 24 hours, blood samples were tested in our laboratory. Plasma was separated by centrifugation at 2000 rpm for 5 min, and pellet containing red blood cells were washed thrice with cold phosphate buffered saline (75 mM PBS, pH 7.4) and finally 4% RBC suspension was prepared. Trypsin was added to a final concentration of 100 µg /ml (0.1 ml of trypsin solution to 1ml of erythrocytes suspension) and incubated for 1 hour at 37°C. The trypsinized RBCs were washed four to five times with PBS and finally 10% suspension was prepared. Percent hemolysis was determined as discussed above.

Results

RESULTS

Hemagglutinating activity of brain homogenate

Buffalo brain extract readily agglutinated trypsinized rabbit erythrocytes. The best solubilizing buffer was found to be 75mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl, 150 mM lactose, and 5 mM β-mercaptoethanol (β-ME). Lactose was an essential competing saccharide in aqueous buffer for efficient solubilization. Moreover, the presence of reducing agent was critical during the purification procedure. If β-ME was eliminated from the aqueous buffer, no lectin activity was recovered. The lectin activity was unaffected by heat treatment up to 45°C for 30 minutes. However, the activity was considerably reduced by the addition of high concentration (over 0.5 M) of sodium chloride. The lectin mediated hemagglutination of rabbit erythrocytes was inhibited by lactose at concentration of 5 mM but glucose and mannose had no effect even at a concentration of 200 mM.

Isolation and purification of β-galactoside binding lectin

Purification

Crude buffalo brain extract (150 g) solubilized in 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl, 150 mM lactose, 5 mM β-ME, 10 mM EDTA and 0.02% sodium azide (w/v). BBL was further purified by a combination of ammonium sulphate fractionation and gel permeation chromatography on sephadex G_{50-80} (1.8 × 59 cm) column. A 40-70% ammonium sulphate fractionation resulted in about 18% yield with five-fold purification with respect to soluble proteins in the homogenate. The high salt content was removed by extensive dialysis against 75 mM sodium phosphate buffer, pH 7.2 containing 30 mM lactose, 0.15 M NaCl and 5 mM β-ME. Lactose was added to the dialyzed sample before loading on to the column to inhibit the lectin and sephadex gel interaction. The salt fractionated protein was further chromatographed on sephadex G_{50-80} equilibrated with same buffer. Fig.6 represents the elution profile with a well defined peak for the protein fraction containing the maximum lectin activity. The results of purification of 150 gm brain are shown in Table III. The yield of the purified protein was 560 μg (from 1350 mg) which represented 71.11% of total activity with fold purification of 1715.35.

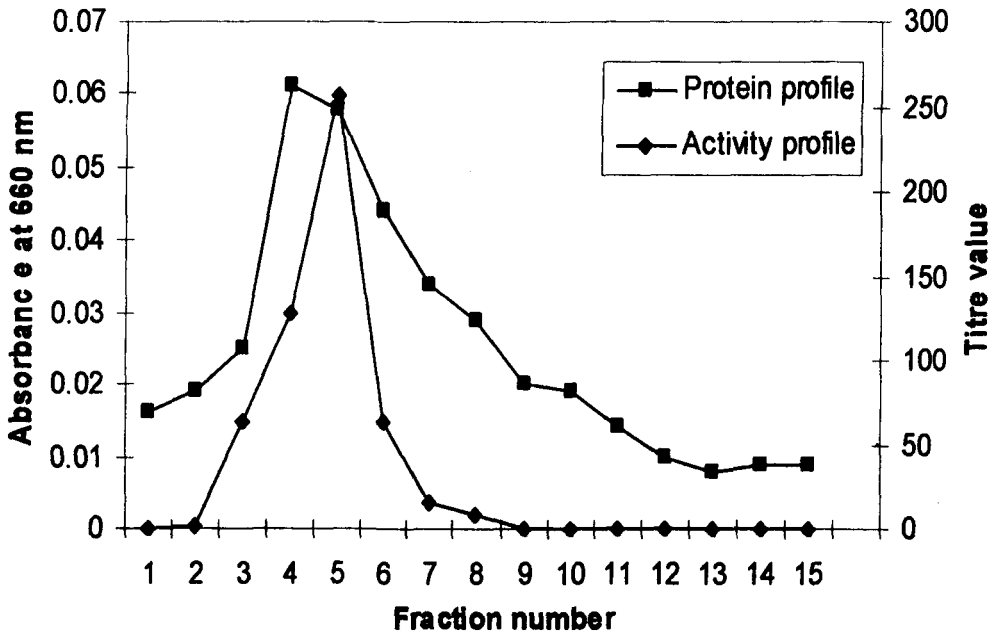


Figure 6. Size exclusion chromatography of BBL

The ammonium sulphate precipitate obtained from 150g of brain homogenate was dissolved and dialysed against several changes of 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl, 5 mM β-ME and 30 mM lactose at 4°C. The sample was applied on sephadex $G_{50\text{-}80}$ column (1.8 × 59 cm), and eluted with same buffer at the flow rate of 20 ml/hr. Fractions of 2 ml were collected and assayed for hemagglutinating activity against trypsinized rabbit erythrocytes and for the protein concentration.

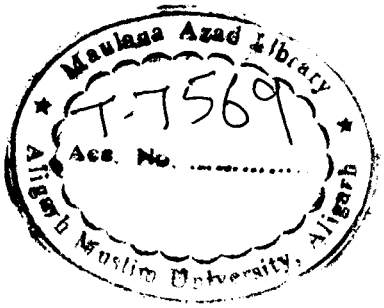


Table III.

Purification of buffalo brain lectin

Step Fraction	Total Volume (ml)	Total protein (mg)[a]	Total activity (Titre)[b]	Specific activity (Titre/mg)	Purification (Fold)	Recovery (%)	Yield (%)
Brain extract	180	1350	14400	10.66	1	100	100
40-70% cut	20	240	12800	53.33	5.00	88.88	17.77
Gel chromatography	2	0.560	10240	18285.71	1715.35	71.11	0.041

Values are mean of three different preparations from 150 gm fresh tissue

[a] Determined by the method of Lowry et al (1951)

[b] The titre of the tested lectin is expressed as the reciprocal of the highest dilution showing agglutination of trypsinized rabbit erythrocytes

Charge homogeneity and Subunit structure

Homogeneity of BBL eluted from gel filtration column was determined by polyacrylamide gel electrophoresis in the absence of SDS. The fraction containing the maximum hemagglutinating activity migrated as a single band in native PAGE (Fig. 7). Subunit structure was determined by SDS-PAGE under non-reducing (in the absence of β-ME) and reducing conditions (in the presence of β-ME) as shown in Fig.8.

Physicochemical characterization of BBL

Molecular weight determination

The subunit molecular weight of BBL under denaturing condition was calculated from its mobility using SDS-PAGE according to the method of Weber and Osborn (1969). Relative mobilities (Rm) of the marker proteins were plotted against the logarithm of molecular weights using least square analysis (Fig.9). The relative mobility of BBL in the presence and absence of reducing agent corresponded to a molecular weight of 14.5 kDa (Fig.10).

The molecular weight of BBL under native condition was determined using gel filtration chromatography on sephadex G_{100} (1.8 × 45 cm) equilibrated with 75 mM PBS, pH 7.2 containing 5 mM β-ME and 30 mM lactose to prevent lectin and gel interaction. The column was calibrated with marker proteins i.e. bovine serum albumin (68 kDa), ovalbumin (45 kDa), soyabean trypsin inhibitor (20.1) kDa and cytochrome c (12.4 kDa). The values of the ratio of elution volume to the void volume (V_e/V_o) for each marker proteins i.e. bovine serum albumin (68 kDa), ovalbumin (45 kDa) soyabean trypsin inhibitor (20.1) kDa and cytochrome c (12.4 kDa) including BBL were calculated as shown in the Table IV. Analysis of data indicated a linear relationship between log M and V_e/V_o. The value of V_e/V_o for BBL was found to be 2.13 which corresponded to a molecular weight of 28.5 kDa (Fig. 11 and Table IV).

Stokes radius

The stokes radius of BBL was determined from its elution volume on sephadex G_{100} column. The column was calibrated by determining the elution volume of several globular proteins with known stokes radii. The data was analyzed according to the theoretical treatment of Laurent and Killander (1964) equation for calculating the stokes radius of BBL under native conditions, and was found to be 25 Å (Fig. 12).

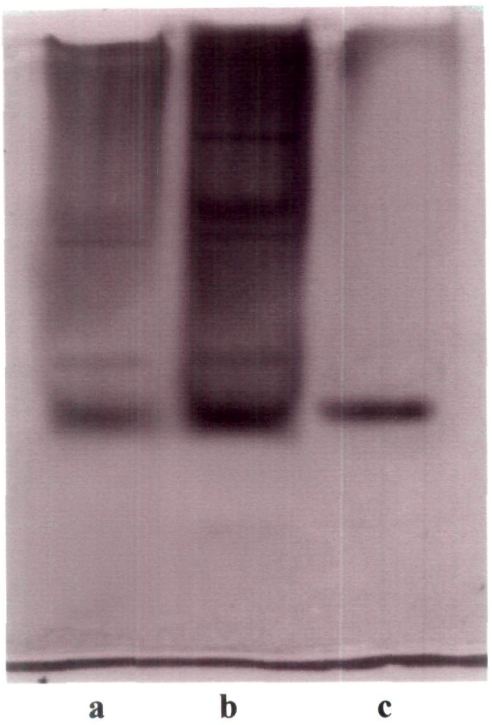


Figure 7. Native electrophoresis of buffalo brain lectin during various stages of purification.

PAGE was performed on 10 % acrylamide gel as described in materials and methods section. Lane (a) contains soluble brain extract (30 μg); Lane (b) contains 40-70% ammonium sulphate fraction (30 μg); Lane (c) contains purified protein (40 μg).

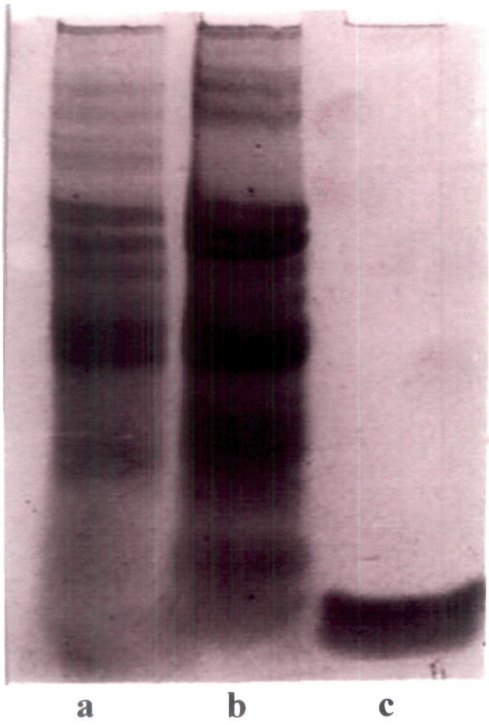


Figure 8. SDS-Gel electrophoresis of buffalo brain lectin during various stages of purification.

SDS-PAGE was performed on 12.5% acrylamide gel under reducing conditions as described in materials and methods section. Lane (a) contains soluble brain extract (30μg); Lane (b) contains 40–70% ammonium sulphate fraction (30 μg); Lane (c) contains purified protein (40 μg).

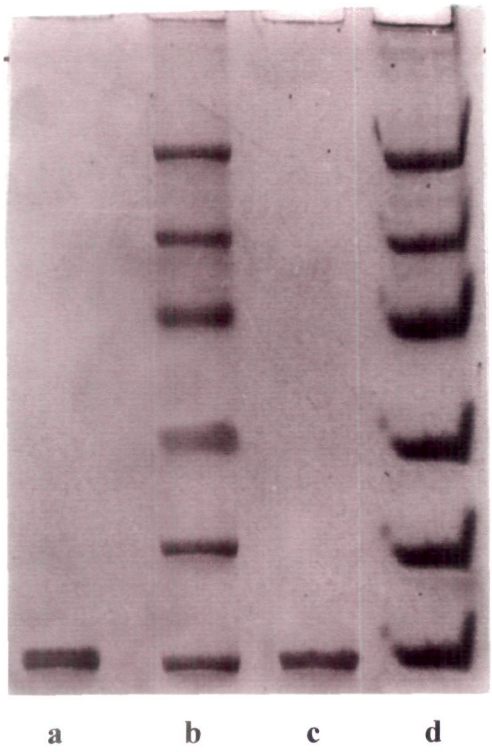


Figure 9. Molecular weight determination of BBL: Samples were separated by 12.5% SDS-PAGE under reducing condition and non-reducing conditions.

Lane (a) purified protein (25 µg) under reducing conditions; Lane (b) molecular weight markers under reducing conditions; Lane (c) purified protein (25 µg) under non-reducing conditions; Lane (d) molecular weight markers under reducing conditions. The molecular weight markers are in the descending order; Phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Soyabean trypsin inhibitor (20.1kDa), Lysozyme (14.4 kDa).

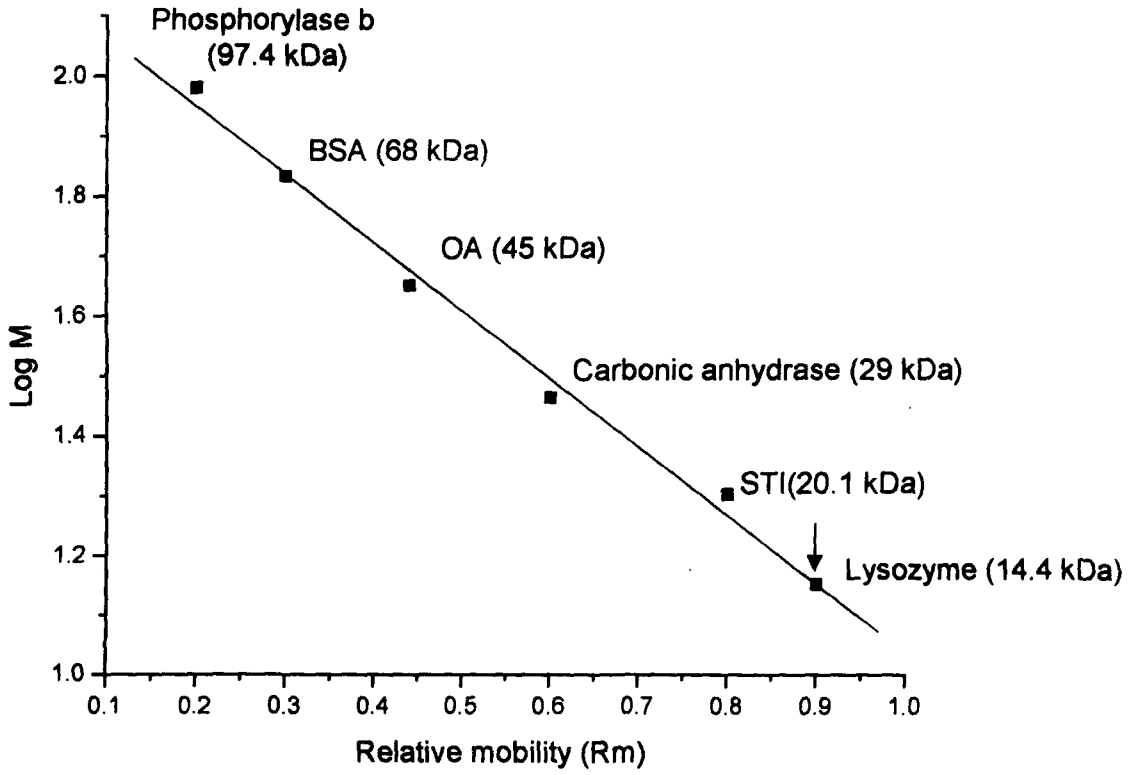


Figure 10. Molecular weight determination of BBL by SDS-PAGE.

Electrophoresis was carried out using 12.5 % SDS-polyacrylamide gel. The relative mobility (Rm) of the marker proteins and BBL were plotted against their molecular weight using least square analysis of the data. Molecular weight of BBL is indicated by an arrow.

Table IV.

Various parameters for marker proteins and buffalo brain lectin calculated from gel filtration chromatography

Marker proteins	Stokes radius (Å)	MW (kDa)	Log M	Elution volume (V_e)	V_e/V_o	$(-\log K_{av})^{1/2}$
Bovine serum albumin	35.5	68	1.82	52	1.40	0.846
Ovalbumin	27.3	45	1.653	64	1.72	0.678
Soyabean trypsin inhibitor	22.6	20.1	1.303	88	2.37	0.430
Cytochrome C	16.4	12.4	1.09	103	2.78	0.273
Buffalo brain lectin	25.0±1.5	28.5±1.2	1.45	79	2.13	0.518

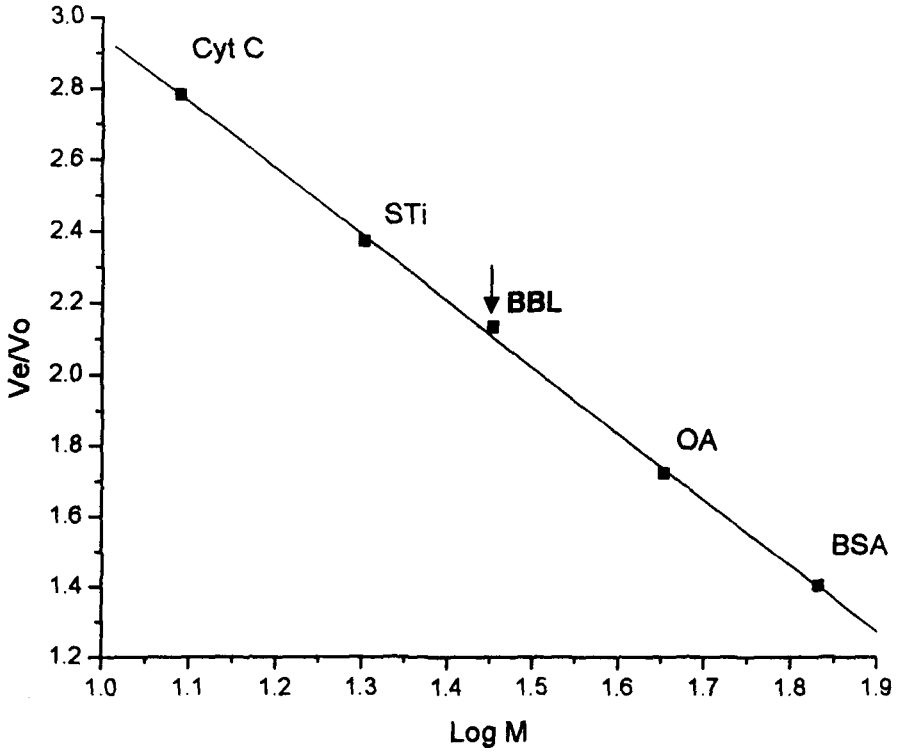


Figure 11. Molecular weight determination of BBL using sephadex G_{100} gel filtration chromatography.

Purified BBL was applied on a column of sephadex G_{100} (1.8 × 45 cm) and eluted with 75 mM PBS, pH 7.2 containing 5 mM β-ME and 30 mM lactose at a flow rate of 15 ml/hr. The molecular weight markers used were bovine serum albumin (BSA 68 kDa), ovalbumin (OA 45 kDa), soyabean trypsin inhibitor (STI 20.1 kDa) and cytochrome c (CC 12.4 kDa). The elution position of the lectin molecule is indicated by an arrow.

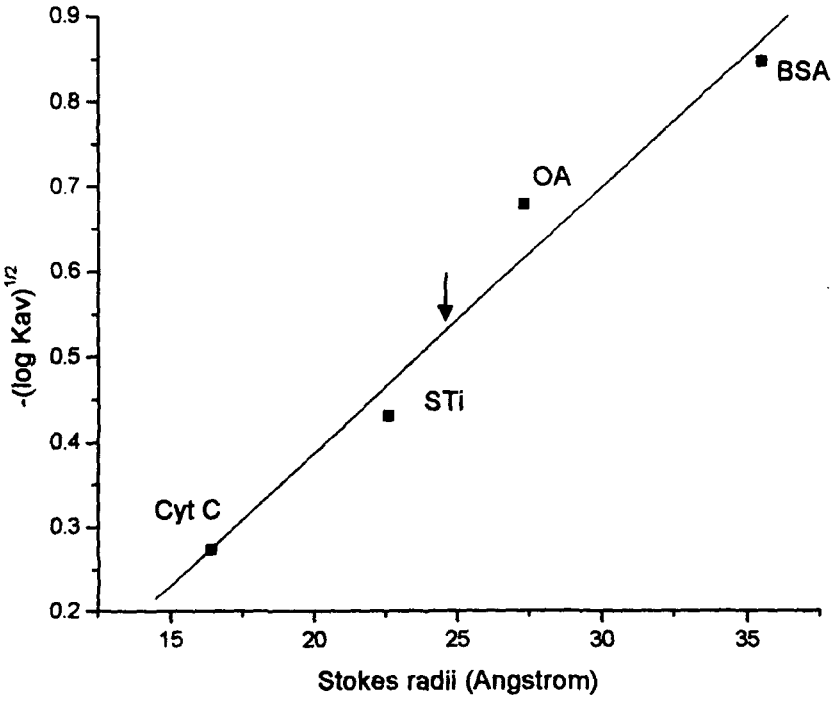


Figure 12. Determination of Stokes radius of the purified BBL by Laurent and Killander plot.

The purified BBL and marker proteins were subjected to gel filtration on sephadex G_{100} column as described in materials and methods section. The Kav values were computed from the elution volumes of marker proteins. Stokes radii for the marker proteins were: 35.5 Å (bovine serum albumin, BSA), 27.3 Å (ovalbumin, OA), soyabean trypsin inhibitor (22.6 Å, STi), and cytochrome C (16.4 Å, Cyt C). Stokes radius of BBL is indicated by an arrow.

Diffusion Coefficient

The diffusion coefficient, D, of BBL corresponding to the value of stokes radius was computed to be 8.91 x 10^{-15} cm^2/s with the help of the equation, $D=KT/6\pi\eta r$, where, K is the Boltzman constant (1.386 x 10^{-16} erg/deg), T is the absolute temperature (303 K) and η is the coefficient of viscosity of the medium [0.0100 P for water and dilute aqueous salt solutions at 20° C (Freidfelder, 1982)].

Carbohydrate binding specificity

The carbohydrate binding affinities of BBL was studied by investigating the effect of various sugars and their derivatives on BBL mediated hemagglutination of trypsinized rabbit erythrocytes. The inhibitory effect of saccharides was tested by using 4 hemagglutinating unit of BBL. Increasing concentration of a specific saccharide caused substantial decrease in hemagglutination. The most potent inhibitor of BBL was lactose giving complete inhibition of hemagglutination at 0.78 mM followed by p-nitropheyl-β-D-galactopyranoside (50mM), galactose (100mM), methyl-β-D-galactopyranoside (100mM) and methyl-α-D-galactopyranoside (100mM) as shown in Fig. 13 and Table V. Other sugars such as D-galactosamine, p-nitropheyl-α-D-galactopyranoside, D-glucose, L-fucose, sucrose, D-mannose, melibiose, and D-fructose were unable to inhibit the lectin mediated hemagglutination even at a concentration of 200mM.

Blood group specificity

The ability of purified BBL to agglutinate human erythrocytes (Blood groups A, B, and O) was assayed using trypsinized as well as untrypsinized preparations of red blood cells. The BBL was titrated against erythrocytes showing positive agglutination using microtitre plate assay. The results are shown in Table VI. The concentration of brain lectin required for agglutination varied markedly with the type of cells. The rabbit erythrocytes treated with trypsin were most sensitive requiring only 1-1.5 μg/ml of lectin for complete agglutination, while the concentration required for trypsinized human erythrocytes was rather high (10-15 μg/ml). BBL agglutinated native human erythrocytes with marked preference for the blood group A, while trypsinized human erythrocytes were found to be more sensitive towards buffalo brain lectin in the order of blood group A=O>B.

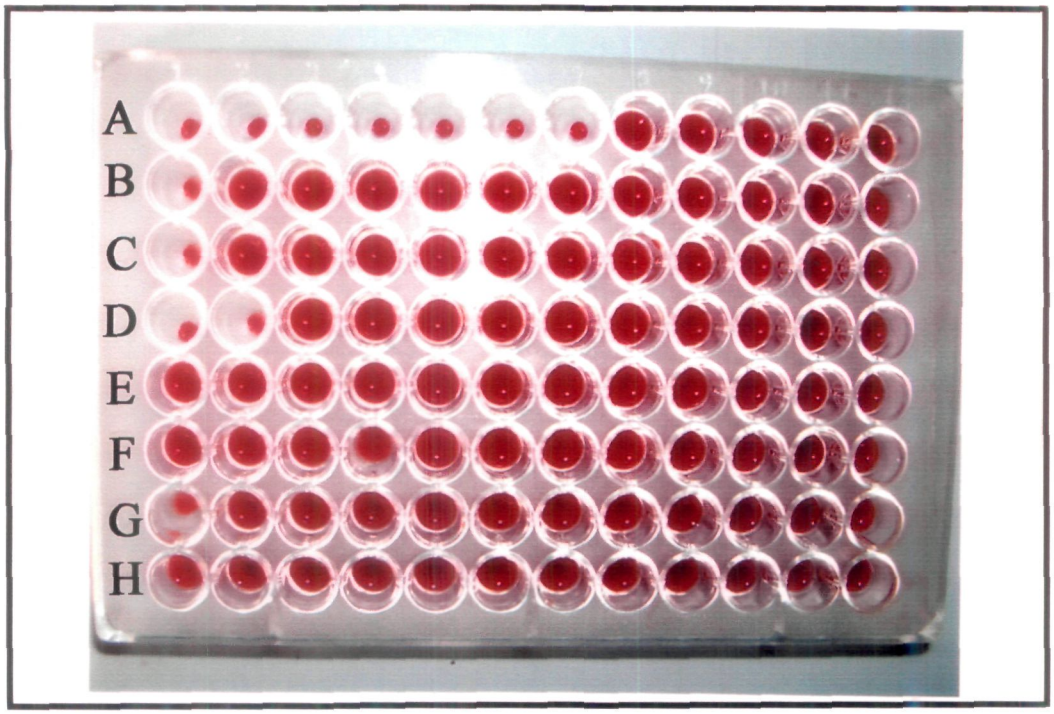


Figure 13. Inhibition of hemagglutination activity of BBL due to various saccharides.

Each well contained 50 µl of serially diluted tested sugar, 50 µl of lectin (4 agglutinating unit) and 50 µl of 4 % suspension of trypsinized rabbit erythrocytes. The minimum inhibitory concentration was obtained by dividing the starting concentration of the carbohydrates i.e. A (lactose (100mM)), B (galactose (200mM)), C (methyl-β-D-galactopyranoside (200mM)), D (p-nitrophenyl-β-D-galactopyranoside (100mM)), E (p-nitrophenyl-α-D-galactopyranoside (200mM)), F (D-galactosamine (200mM)), G (Methyl-α-D-galactopyranoside (200mM)), H (glucose (200mM)) by the reciprocal of the highest inhibitory dilution, taking into account that due to the addition of BBL, the dilution of the test sugar is 1:2 in the first well.

TABLE V.

Effect of various saccharides on the hemagglutinating activity of BBL

Carbohydrates	Minimum concentration of sugar giving complete hemagglutination inhibition*
Lactose	0.78
Galactose	100
Methyl-β-D-galactopyranoside	100
p-nitrophenyl-β-D-galactopyranoside	50
p-nitrophenyl-α-D-galactopyranoside	Nil
D-galactosamine	> 100
Methyl-α-D-galactopyranoside	100
Glucose	Nil

The following saccharides were also tested and showed no inhibitory activity even at 200mM concentration: *D*- mannose, *L*-fucose, sucrose, melibiose, and D-fructose.

*Each value represents the mean of three independent experiments performed in triplicates.

TABLE VI.

Human blood type specificity of buffalo brain lectin

Blood type	Titre	
	Untrypsinized	Trypsinized
A	8	32
B	4	16
O	8	32

Titre* .The reciprocal of the highest dilution giving visible hemagglutination
Four hemagglutinating units per 50 µl were taken in each assay; the results shown are mean of three different preparations taken in triplicates.

Determination of binding parameters of lactose with lectin by equilibrium dialysis

The Scatchard plot of the equilibrium dialysis data of binding of BBL in 75mM PBS pH 7.2 containing 5mM β-ME with lactose (40-400 μM) at 37°C is shown in Fig.14. A linear plot was obtained, the slope of which gave the binding constant K_{ass} as 6.66 x 10^3 M^{-1}. The number of binding sites calculated from the X intercept was found to be 1.7 per lectin dimer suggesting the presence of one binding site per subunit.

Carbohydrate moiety

BBL was found to contain 3.3% carbohydrate content, which may correspond to 3 residues of carbohydrate per lectin molecule.

Sulphydryl groups

Thiol group analysis of the lectin indicated a molar ratio of 2.8 suggesting the presence of three-sulphydryl groups per mole of protein.

Thermal stability

BBL retained full activity when maintained at temperatures up to 45°C (Fig. 15). A gradual decrease of activity was observed when it was heated between 45-65°C, while a sharp decline was observed at 70°C.

pH stability

The BBL showed maximum hemagglutinating activity at pH 7.5, with stability between 6.5-9.5 (Fig. 16).

Functional modification of lectin with alkylating agents

The extracted lectin showed activity only in the presence of reducing agents like β-ME and dithiothreitol. To examine the role of thiol groups in the sachharide binding, these groups were modified by iodoacetate and iodoacetamide. Treatment of alkylating agents resulted in a considerable loss of lectin activity, which was studied by measuring the time course of inactivation by 70 mM each of iodoacetate and iodoacetamide in 75 mM PBS, pH 7.2 containing 1mM β-ME (Fig. 17). Iodoacetamide inactivated more than 75 % activity of buffalo brain lectin within 20 minutes but no further significant loss of activity was observed, whereas, iodoacetate showed a much gradual decrease in the activity. This indicated the possible role of thiol groups of lectin in the saccharide binding and the need of a reducing agent to maintain the active form of lectin.

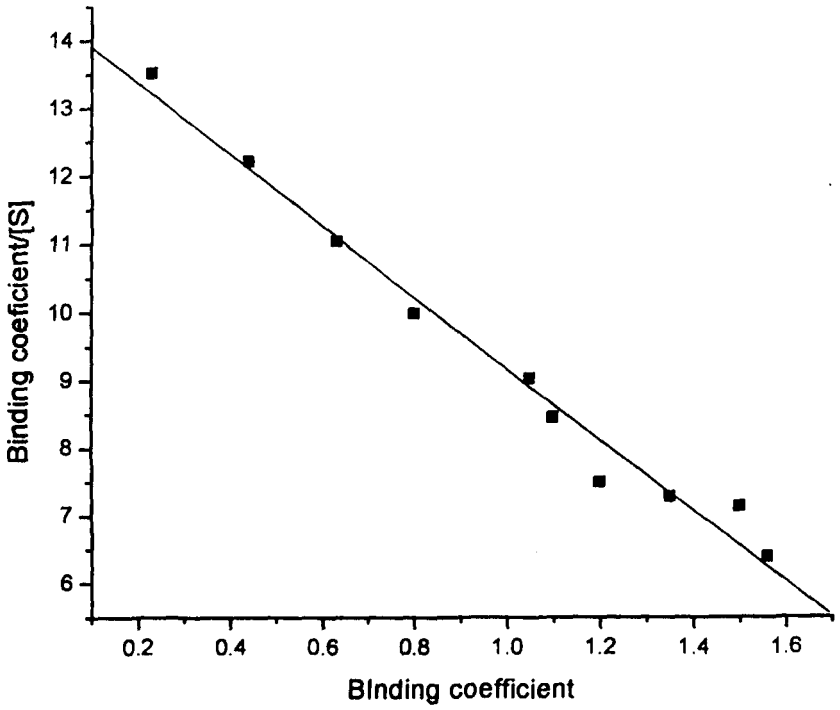


Figure 14. Scatchard plot for the binding of lactose to BBL.

A fixed concentration of BBL (100μM) in 75 mM sodium phosphate buffer pH 7.2 containing 0.15 M NaCl and 5 mM β-ME in a dialysis bag was incubated with 40-400 μM of lactose in the same buffer at 37°C for 24 hrs. After the attainment of equilibrium the decrease in lactose concentration was estimated in the dialysate. Analysis of results yielded the value of association constant and the number of sugar binding sites.

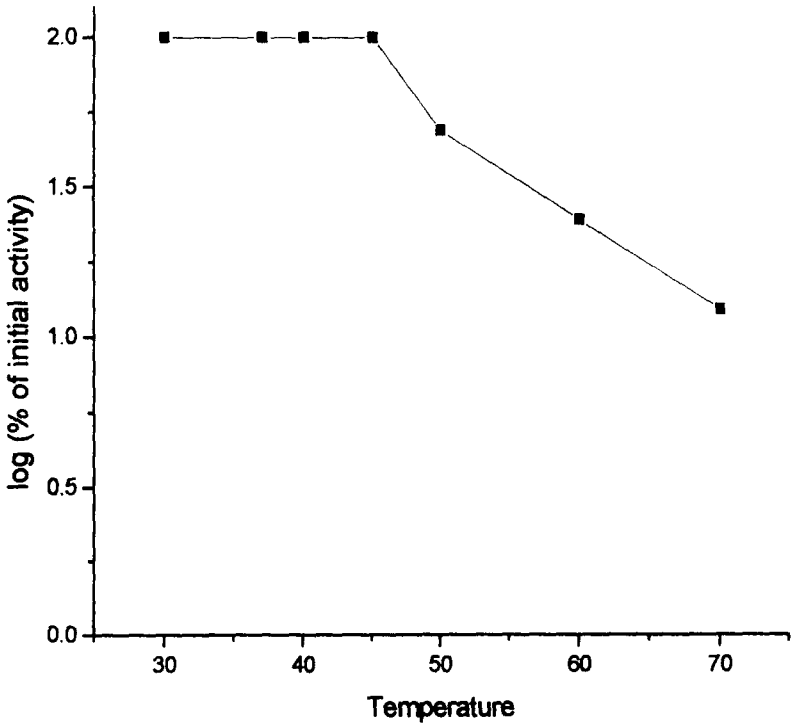


Figure 15. Thermal stability of BBL.

Buffalo brain lectin (125μg/ml) was incubated for 30 min at various temperatures (30-70 °C). The hemagglutinating activity was measured by microtitre plate assay and the initial activity (100%) refers to the activity of the unincubated lectin.

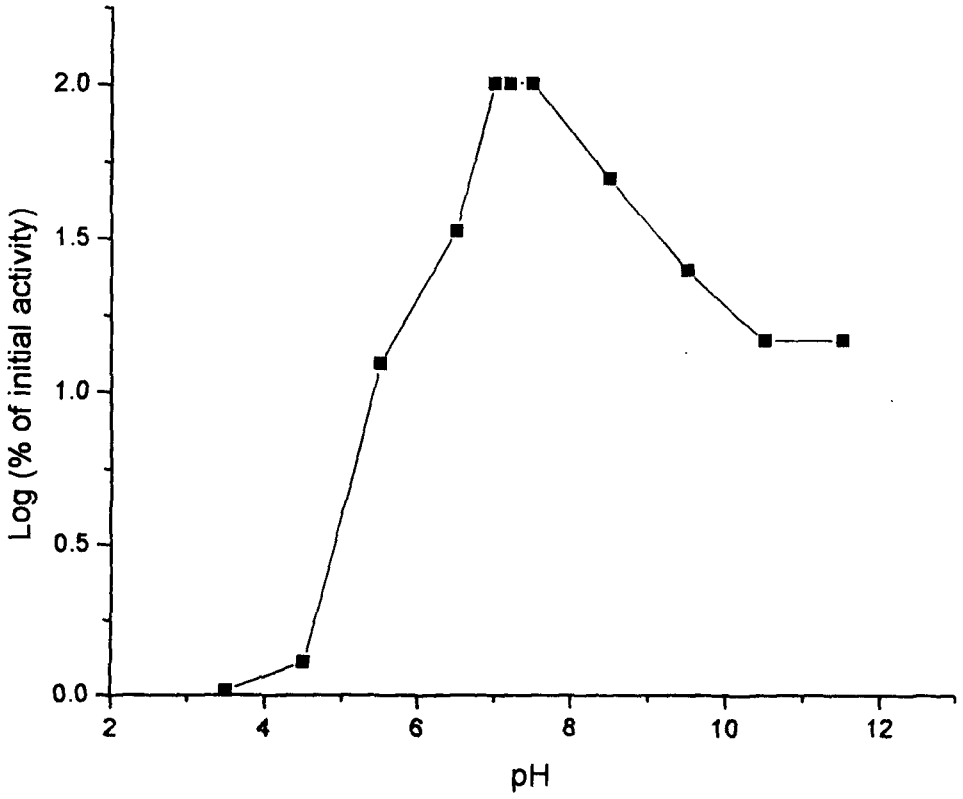


Figure 16. The pH dependence of BBL.

Native BBL (125 μg/ ml) were incubated in various buffers (0.1 M sodium acetate/sodium phosphate/Tris-HCl/ glycine NaOH) of different pH values (3.5-11.5) at 4°C for 24 hours. Hemagglutinating activity was assayed by microtitre plate assay. Initial activity refers to the activity of BBL in normal saline containing 5 mM β-ME.

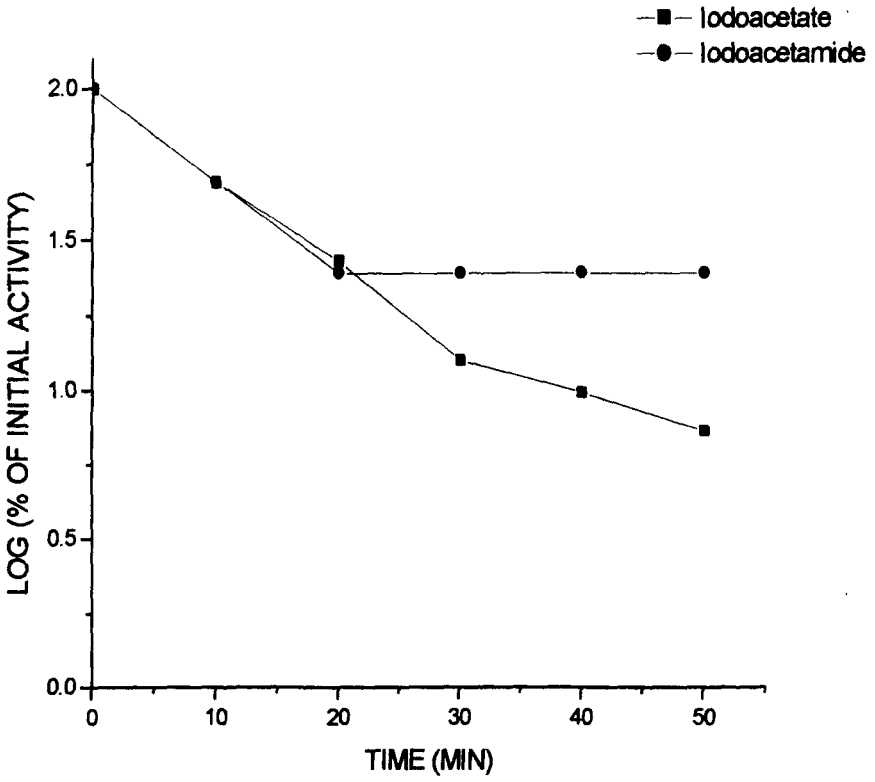


Figure 17. Effect of alkylating agents on the activity of BBL.

Buffalo brain lectin (125 μg/ml) having 256 hemagglutinating units (100% activity) was incubated at room temperature with 70 mM iodoacetate or iodoacetamide in 75 mM of sodium phosphate buffer containing 0.15 M NaCl and 1 mM β-ME pH 7.2 at 37°C for different time intervals (0-50 min) and residual activity was measured by microtitre plate assay. The initial activity refers to the hemagglutinating activity of BBL in the absence of the alkylating agents.

Effect of detergents

The effect of increasing concentration of SDS on the activity of native lectin is shown in Fig. 18. The activity of the lectin showed a steep decline with loss of 50 % activity at 0.5 mg % SDS. Complete inhibition was achieved at 2 mg % of SDS concentration. However, when native protein was treated with SDS in the presence of 10 mM lactose, effective protection was demonstrated against denaturation. Pre-incubation of BBL with varying concentration of non-ionic detergents i.e. Tween-20 and Triton X-100, resulted in a lesser loss of lectin activity (Fig. 19). Addition of Tween-20 showed a decline in the activity with 50 % decrease at 5 % (v/v) of Tween 20. Similarly Triton X-100 lowered the agglutination activity of native lectin with reduction of 66.6 % at 5.0 % (v/v) Triton X-100 concentration. BBL pre-treated with 10 mM lactose did not show any change in the activity at all inhibiting concentrations of Tween-20 and Triton X-100.

Effect of metal ions

Addition of metal cations ($CaCl_2$, $MnCl_2$, $[Sr(CHCOO)_2]$, $MgCl_2$, $NiCl_2$) showed no effect on hemagglutinating activity, suggesting that BBL activity was not dependent on metal ions.

Brain aggregation assay

The purified BBL preferentially agglutinated buffalo brain cells (75%; 45 x 10^4 cells/ml) than goat brain cells (63%; 38 x 10^4 cells/ml) (Fig. 20). Optimum pH and temperature for brain cell aggregation activity of BBL was found to be 7.5 and 40°C, respectively (Fig 21, 22). The optimal pH and temperature did not change for goat brain cells and was same as that for buffalo brain cells.

Spectroscopic properties of lectin

UV spectra

Ultraviolet absorption spectrum of purified protein was studied in 75 mM PBS pH 7.2, containing 5 mM β-ME in the wavelength of 220-320 nm. The lectin displayed an absorption maximum at 282 nm, and a minimum at 250 nm corresponding to the presence of single tryptophan residue and a large number of other aromatic residues (Fig.24). When excited at 280 nm, the native lectin showed fluorescence emission spectrum with a maximum at 335nm, typical of a tryptophan residue in a hydrophobic environment as shown in Fig. 25.

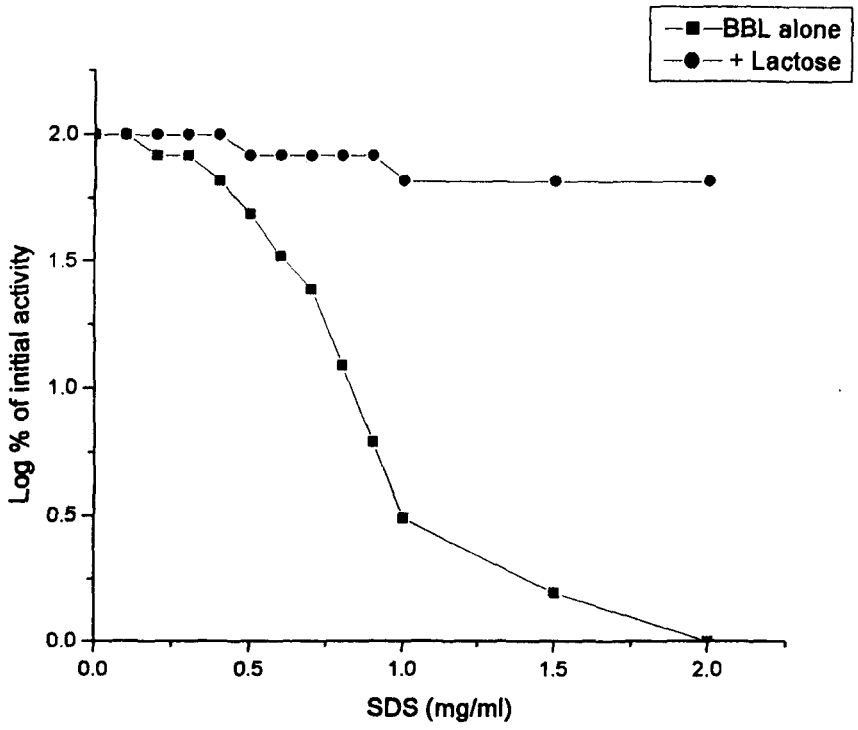


Figure 18. Effect of increasing concentrations of SDS on the activity of BBL.

BBL (125 μg/ ml) having initial titre value of 256 (100% activity) was incubated with varying concentrations of SDS (0.1-2 mg/ml) in 75 mM of sodium phosphate buffer containing 0.15 M NaCl and 5 mM β-ME pH 7.2 at 37°C for one hour. The residual activity was measured by microtitre plate assay. The initial activity refers to the hemagglutinating activity of the unincubated protein.

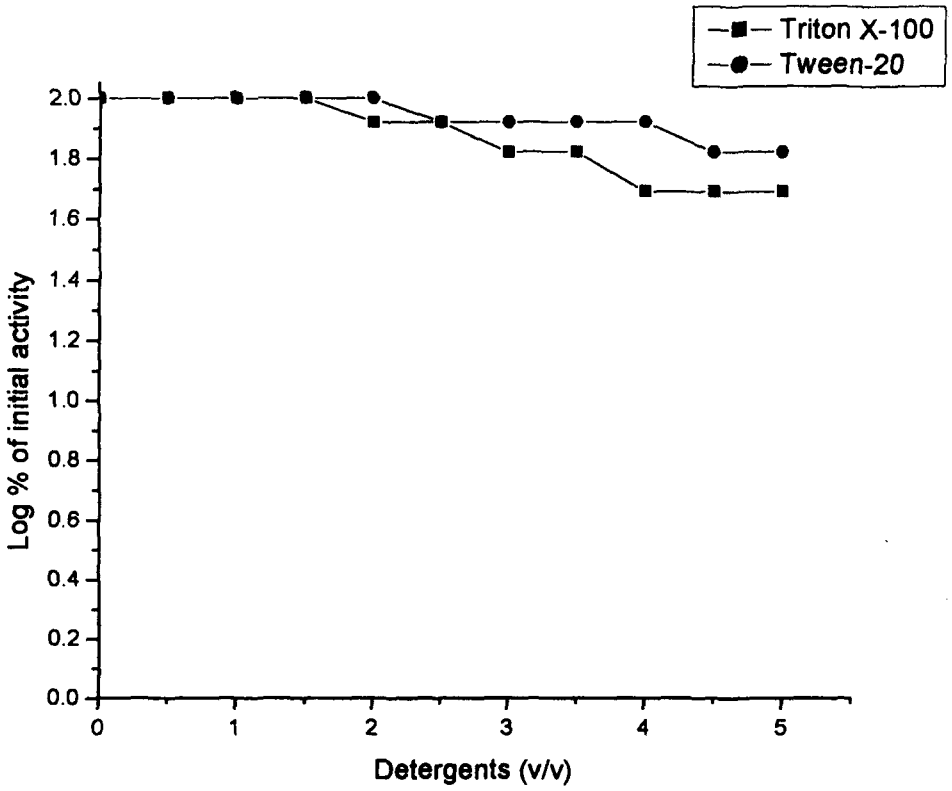


Figure 19. Effect of increasing concentrations of Tween-20 and Triton X-100 on BBL.

BBL (125 μg/ ml) with initial titre value of 256 (100% activity) was incubated in varying concentrations of Tween-20 and Triton X-100 (1-5% v/v) in 75 mM of sodium phosphate buffer containing 0.15 M NaCl and 5 mM β-ME pH 7.2 at 37°C for one hour. The residual activity was measured by microtitre plate assay. The initial activity refers to the hemagglutinating activity of the unincubated protein.

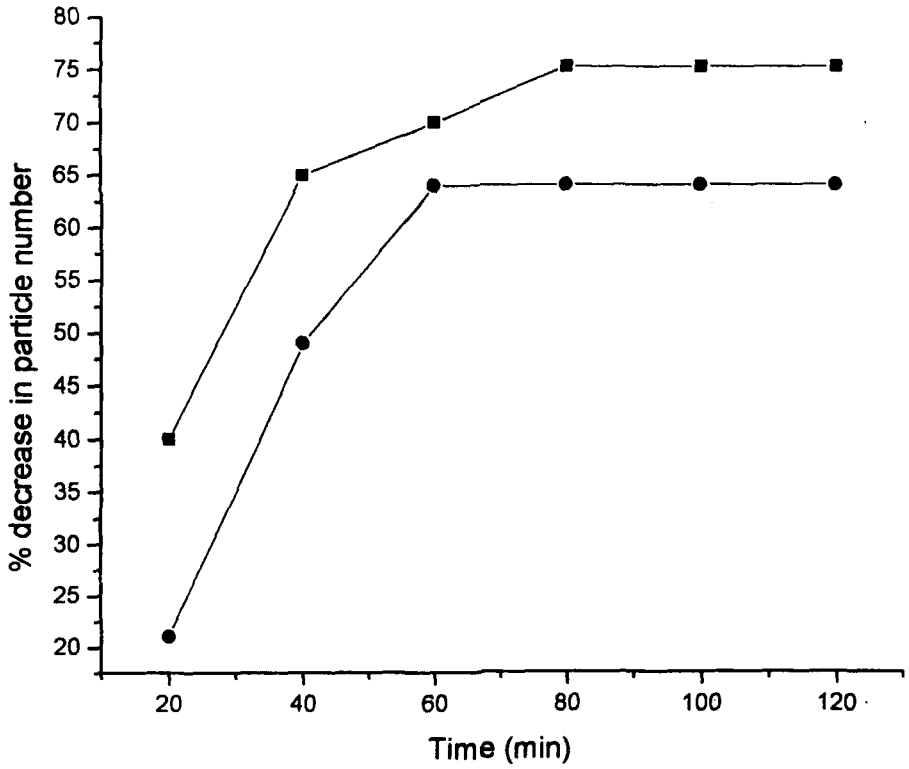


Figure. 20. Brain cell aggregation by BBL

BBL induced aggregation of brain cells from adult buffalo (■) and goat (●) were detected by measuring the decrease in the number of total free particles by hematocytometer.

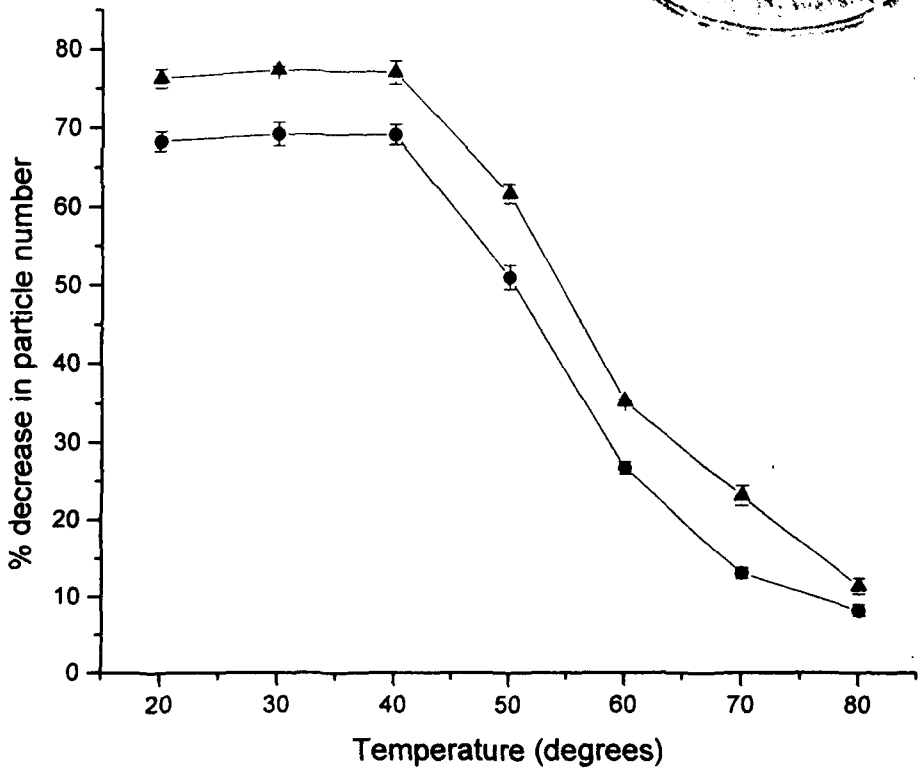


Figure 21. Effect of temperature on brain cell aggregation by BBL

BBL induced aggregation of brain cells from adult buffalo (▲) and goat (●) were detected by measuring the decrease in the number of total free particles by hematocytometer after incubating at different temperatures (20-80 °C) for 30 min.

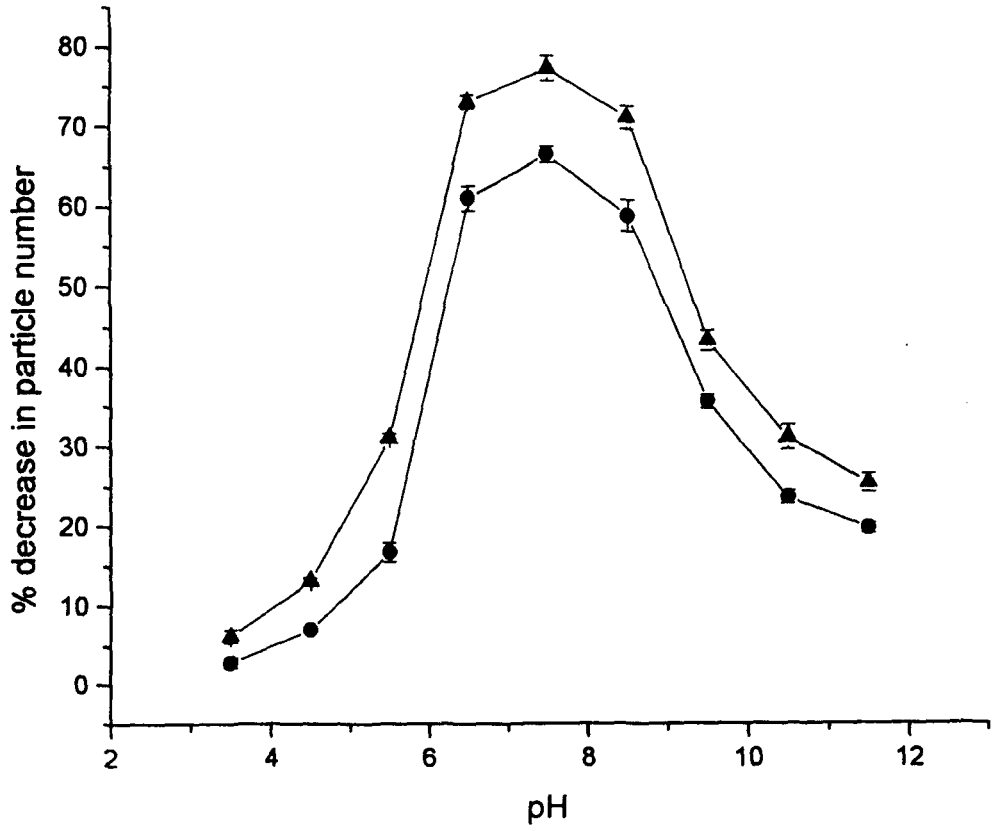


Figure 22. Effect of pH on brain cell aggregation by BBL.

BBL induced aggregation of brain cells from adult buffalo (▲) and goat (●) were detected by measuring the decrease in the number of total free particles by hematocytometer after incubating in various buffers (0.1 M sodium acetate/sodium phosphate/Tris-HCl/ glycine NaOH) of different pH values (3.5-11.5) at 4°C for 24 hours.

Circular dichroism Spectra

The far-ultraviolet circular dichroism spectra of native lectin in 10 mM sodium phosphate buffer pH 7.5 are shown in Fig. 27. A low intensity spectrum was observed with a minimum in the range between 215-217 nm consistent with the large extent of β-sheet structure profile. The α-helical structure was found to be absent in BBL due to the absence of trough at 222 nm and 208 nm (characteristic feature of α-helix). The CD-spectra of BBL in the near UV range is depicted in Fig. 29 which gives a picture of tertiary structure of BBL.

Modification of lectin in the presence of H_2O_2 and lactose

The time course of inactivation of buffalo brain lectin in the presence of H_2O_2 was measured in 75 mM PBS pH 7.2 containing 1 mM β-ME. The exposure of BBL to H_2O_2 showed a remarkable decrease in the hemagglutination activity with time (Fig.23). Functional modification of BBL in the presence of oxidizing agent was due to the conformational changes in the native structure of BBL. This was confirmed as UV spectrum (Fig.24) of native BBL showed a sharp decline in the maxima from 282 to 250 nm in the presence of H_2O_2 typical as that of oxidation of tryptophan residue. Fluorescence spectroscopy shows a quenching of the intrinsic fluorescence of the native spectra in the presence of 5 mM of H_2O_2 as is evident in fluorescent profile (Fig. 25). The peak position of oxidized protein also showed a blue shift from 335 to 332 nm accompanied by quenching of fluorescence intensity. Fig. 26 shows a rate of change of fluorescence intensity in the presence of 5 mM H_2O_2. This decrease of fluorescence intensity can be compared to the decrease in the activity profile and can be assessed that both the changes are concomitant to each other. When fluorescence experiments of the native protein were carried out in the presence of 0.1 M lactose solution a large enhancement in the fluorescence intensity was observed, suggesting that the flourophore is possibly present in the vicinity of the lactose-binding site. Exposure of lectin to 5 mM H_2O_2 in the presence of 0.1 M lactose showed less decrease of fluorescence (Fig.25) as compared to the decrease in its absence. The changes in the secondary structure of the native BBL in the presence of H_2O_2 was also analyzed by CD and fluorescence tagged infra red (FTIR) spectroscopy analysis. The far-ultraviolet circular diochroism spectra (Fig. 27) of native BBL in 10 mM sodium phosphate buffer pH 7.2 containing 0.15 M NaCl and 5 mM β-ME revealed a low intensity spectrum with a minimum in the range between 215-217 nm. The CD-

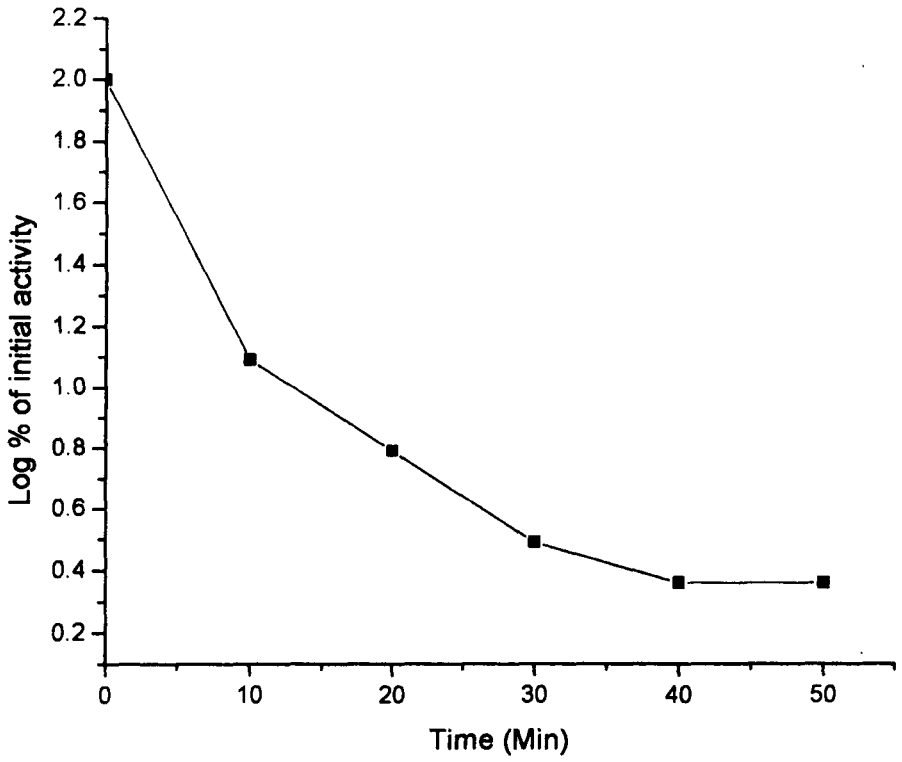


Figure 23. Effect of oxidant on the hemagglutination activity of BBL.

Buffalo brain lectin with titre value of 256 (80 μg/ml) was incubated with 5mM H_2O_2 at room temperature for different time intervals (1-50 min) and residual activity was measured by microtitre plate assay. Initial (100%) activity refers to the activity of the unincubated lectin.

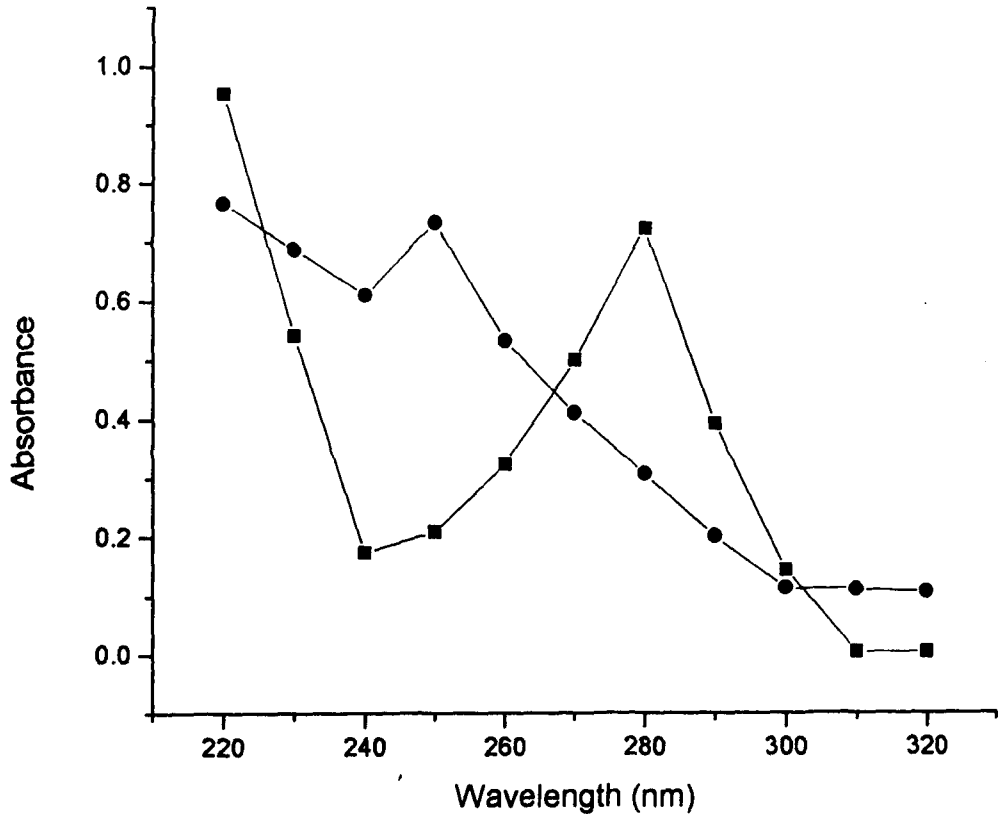


Figure 24. Ultraviolet spectra of native and oxidized BBL.

Spectra of native buffalo brain lectin (150μg/ml) in 75 mM PBS, pH 7.2 containing 5 mM β-ME (■) and after adding 5 mM H_2O_2 in the absence of β-ME (●).

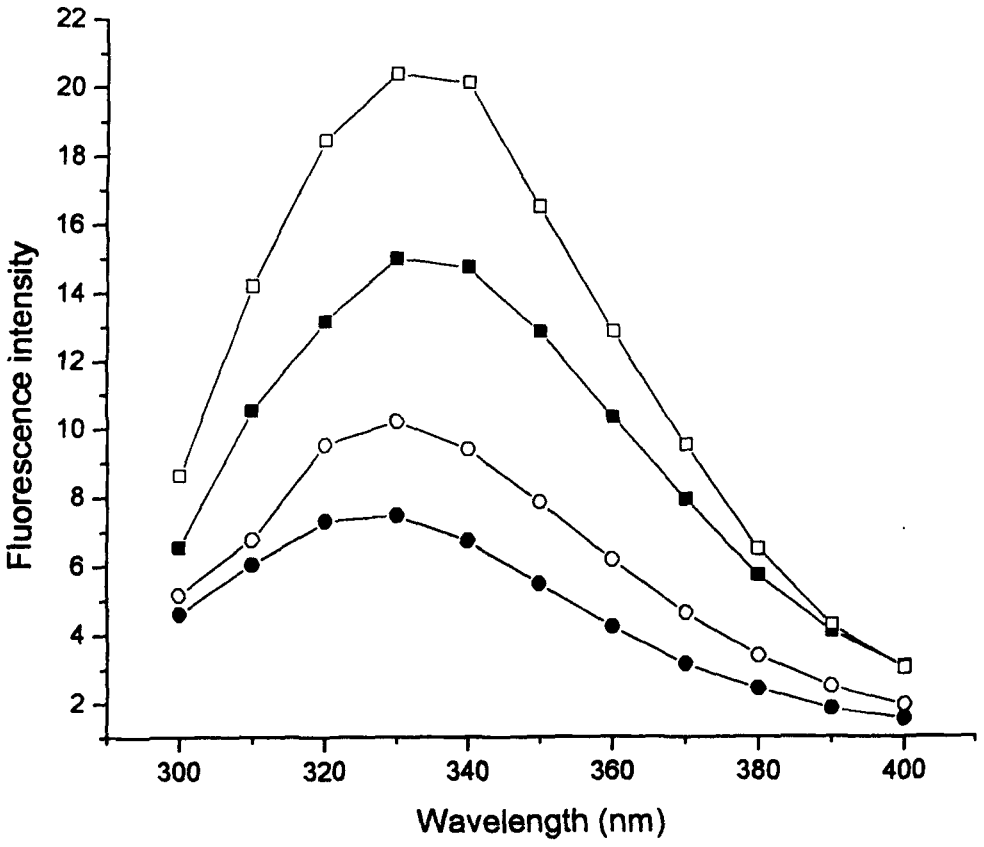


Figure 25. Fluorescence spectra of BBL.

Experiments were performed with BBL (45 μg/ml) in 75 mM sodium phosphate buffer pH 7.2. The spectra of native BBL alone (■), in the presence of 0.1 M lactose (□), oxidized BBL alone (by adding 5 mM H_2O_2) (●) and oxidized BBL in the presence of 0.1 M lactose (○) were measured between 300-400 nm.

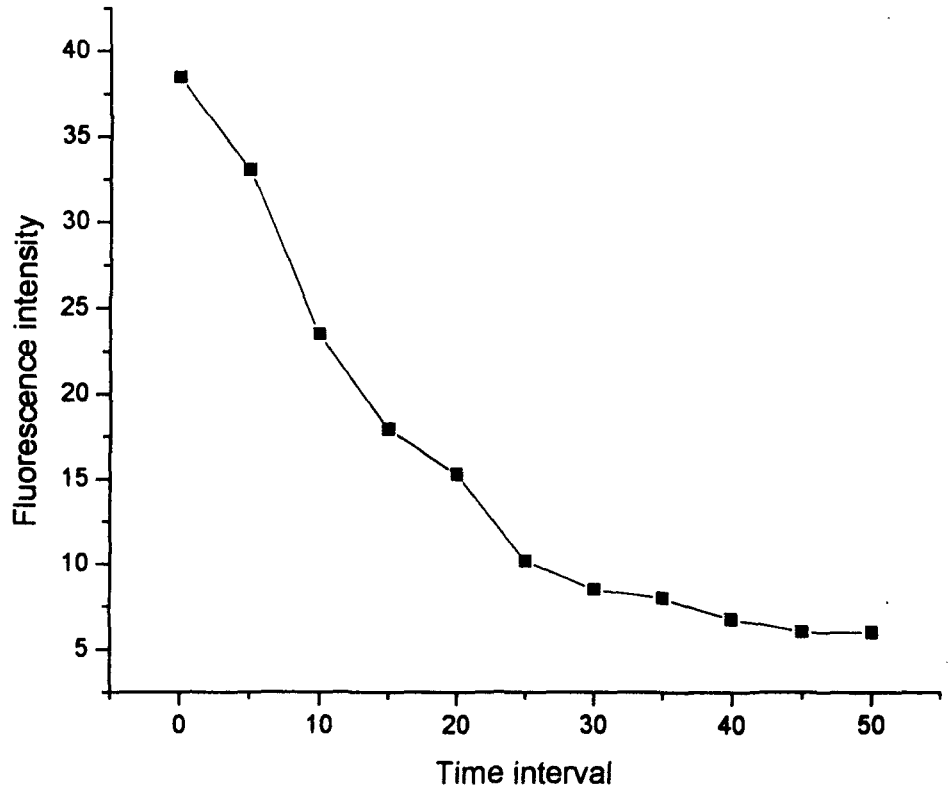


Figure 26. The rate of change in relative fluorescence of BBL.

The rate of change of relative fluorescence of BBL (40μg/ml) at 340 nm on incubation with 5mM H_2O_2 was measured at 22°C after excitation at 280nm

spectra of BBL in the near UV range is depicted in Fig. 29 which gives a picture of tertiary structure of BBL. The effect of H_2O_2 on the secondary (Fig. 28) and tertiary structure (Fig. 30) of native lectin was also studied using circular diochroism spectroscopy. The CD spectral analysis revealed that oxidant exposure caused a remarkable change in the far UV CD spectra with alterations in the β-sheet towards predominantly α-helical structure (characterized by the minima around 208 and 222 nm). Addition of H_2O_2 to the lectin also indicated changes in the near UV CD spectra of the native lectin, shedding light on the significant change in the tertiary structure of the lectin upon oxidation. Addition of lactose alone did not cause any significant change either to the secondary (Fig. 27) or tertiary structure (Fig. 29) of the native lectin as depicted by spectral analysis in far UV and near UV region respectively, while oxidation of BBL pre-incubated with lactose displayed a lesser change in secondary (Fig. 28) and tertiary (Fig. 30) structure of lectin as compared to its native form.

FTIR analysis confirmed the conformational changes during BBL-lactose and BBL-H_2O_2 interactions as shown by circular diochroism studies. In the IR spectra of proteins, the secondary structure is most clearly reflected by the amide I and II bands, particularly the former (Elliot and Ambrose, 1950; Timasheff et al., 1967); the amide I band absorbs at 1657 cm^{-1} (mainly a C=O stretch), and the amide II band absorbs at 1542 cm^{-1} (C-N stretching coupled with N-H bending modes) (Elliot and Ambrose, 1950; Timasheff et al., 1967). It has also been reported that, for a native protein, the amide I component for the α helical structure locates at 1656 ± 2 cm^{-1} and the band components for the β-sheet structure should locate between 1622 and 1642 cm^{-1} (Elliot and Ambrose, 1950; Timasheff et al., 1967). Fig. 31 shows the spectra of the native lectin alone and co-incubated with oxidizing agent and lactose at 37° C. The major peak at 1635 cm^{-1} as shown by native lectin is consistent with the presence of large extent of β-pleated sheet. The presence of lactose did not cause any significant change in the peak position, whereas the presence of 5 mM H_2O_2 caused a major transition of peak from 1635 $cm^{-1}$to 1652 cm^{-1} (characteristic of α-helix). Oxidation induced peak shift from β-pleated to α-helical regions of lactose treated BBL was less as compared to H_2O_2 treated native BBL.

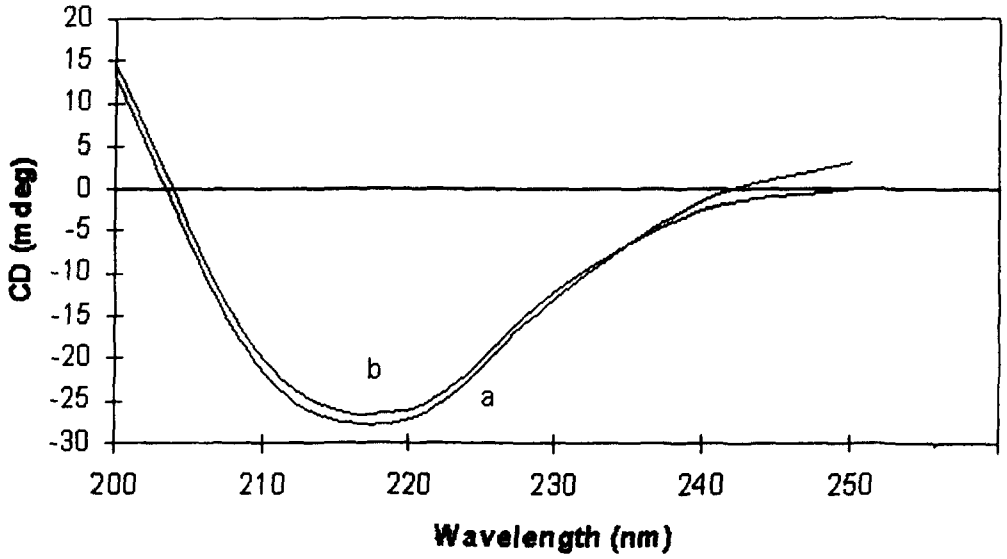


Figure 27. Far- UV-CD spectra of native BBL in the presence of lactose.

The spectra of native BBL (0.250 mg/ml) in 10 mM PBS pH 7.2 was recorded between 200-250 nm using path length of 0.1 cm, in the absence (a); and presence of 0.1 M lactose (b).

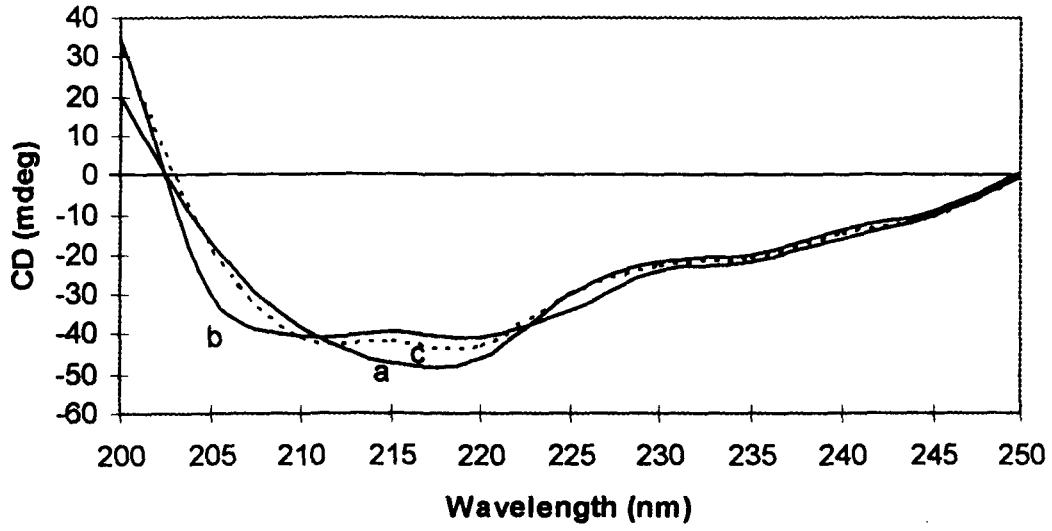


Figure 28. Far-UV-CD spectra of native BBL in the presence of H_2O_2.

The spectra of BBL (0.250 mg/ml) in 10 mM PBS pH 7.2 was recorded between 200-250 nm using path length of 0.1 cm. in the presence and absence of oxidizing agent. (a) native BBL; (b) oxidized BBL (by adding 5 mM H_2O_2 in the absence of β-ME); and (c) oxidized BBL pre-incubated with 0.1 M lactose.

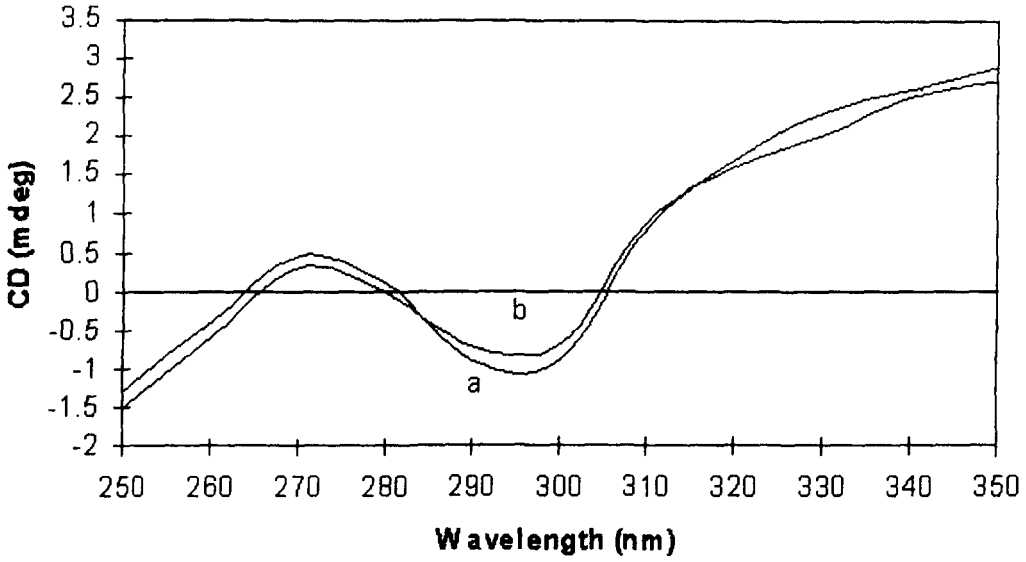


Figure 29. Near- UV-CD spectra of native BBL in the presence of lactose.

The spectra of BBL (1mg/ml) in 10 mM PBS, pH 7.2 was recorded in the absence (a) and presence (b) of 0.1M lactose between 250-350 nm using 0.1 cm path length.

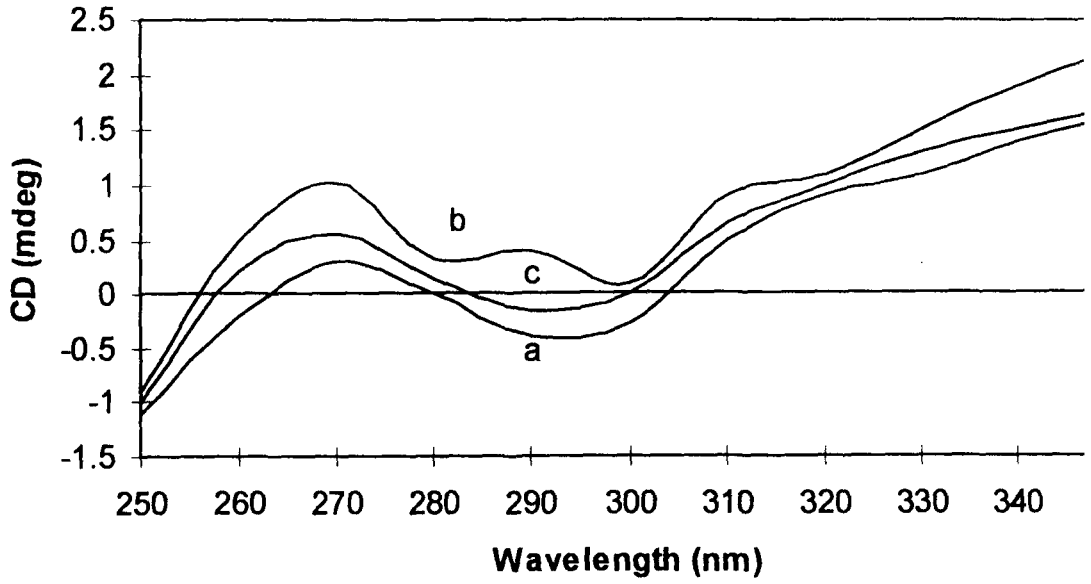


Figure 30. Near- UV-CD spectra of native BBL in the presence of H_2O_2.

The spectra of native BBL (1 mg/ml) in 10 mM PBS pH 7.2 (a); oxidized BBL (by adding 5 mM H_2O_2 in the absence of β-ME) (b); and oxidized BBL pre-incubated with 0.1 M lactose (c), was recorded between 250-350 nm using path length of 0.1 cm.

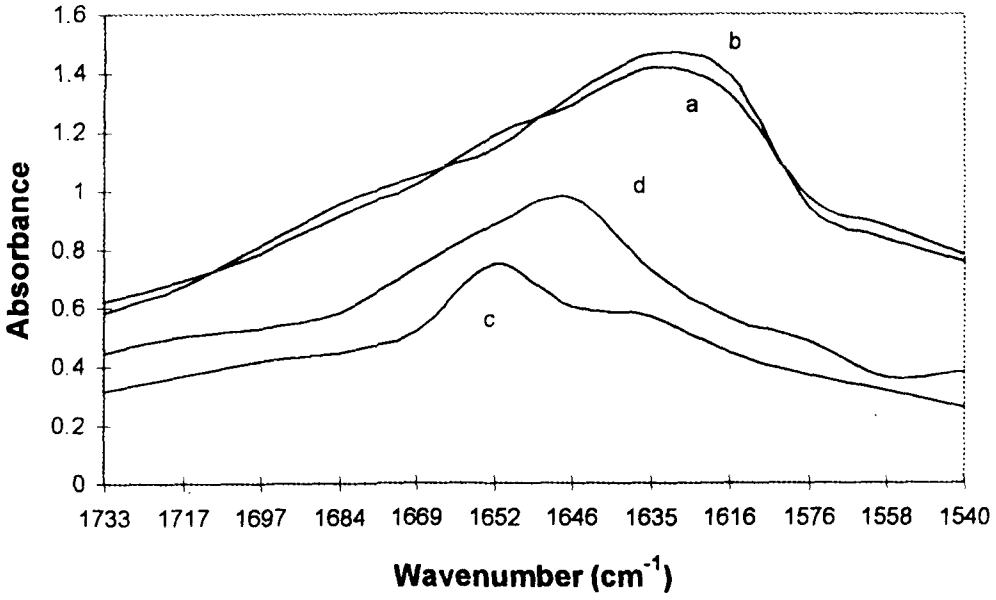


Figure 31. FTIR spectra of BBL.

The spectra of BBL (0.15 mg/ml) in 10 mM PBS pH 7.2 were recorded in the presence and absence of lactose/ H_2O_2. a. native BBL; b. BBL with lactose,; c. oxidized BBL (by adding 5 mM H_2O_2 in the absence of β-ME) d. oxidized BBL pre-incubated with 0.1 M lactose.

Effect of denaturants on the activity and structure of lectin

Activity

Effect of denaturants like GdnHCl, urea and thiourea on the hemagglutinating activity of the native lectin was monitored at varying concentration of the denaturants (0-8 M) followed by incubation for four hours at 37°C. Both GdnHCl and urea caused a significant loss in the titre value with decrease of 50% at 3.0 M concentration, while thiourea reduced the lectin activity to 50% at 4.0 M. There was a leveling of activity of lectin at lower concentration of urea and GdnHCl (Fig. 32).

Conformational change

The denaturation of lectin was also monitored by differential fluorescent behaviour in the presence of varying amounts of denaturants at 37 °C after an incubation period of four hours. The emission spectra were recorded between 300-400 nm wavelengths by exciting the protein at 280 nm. Complete denaturation of protein was observed at 6 M GdnHCl with a 40% decrease in fluorescence intensity and shift of emission maxima from 340 to 357 nm (Fig. 33). Fluorescence intensity profile of BBL in the presence of urea depicted an increase in intensity, and complete unfolding was observed at 8 M urea with a 30.8 % increase in fluorescence intensity and shift of emission maximum from 337 to 355 nm (Fig.34). Thiourea caused a similar increase in the fluorescence intensity with a 25.8 % increase in fluorescence intensity with a red shift from 337 nm to 360 nm (Fig.35).

Deglycosylation of lectin and its effect on its stability

Deglycosylation

The method applied for the deglycosylation completely removed the carbohydrate moiety from the native glycosylated BBL which was confirmed by phenol-sulphuric acid method (Dubois et al., 1956). The extent of deglycosylation was also monitored by means of SDS-PAGE (Fig.36) where the migration of native BBL was compared to deglycosylated protein. We found that periodate treatment of glycosylated lectin resulted in complete removal of sugar residues as the mobility of deglycosylated protein has increased and molecular weight was decreased to 14.0 kDa (Fig. 36). The effect of deglycosylation on the molecular weight of lectin was also investigated by gel filtration analysis.

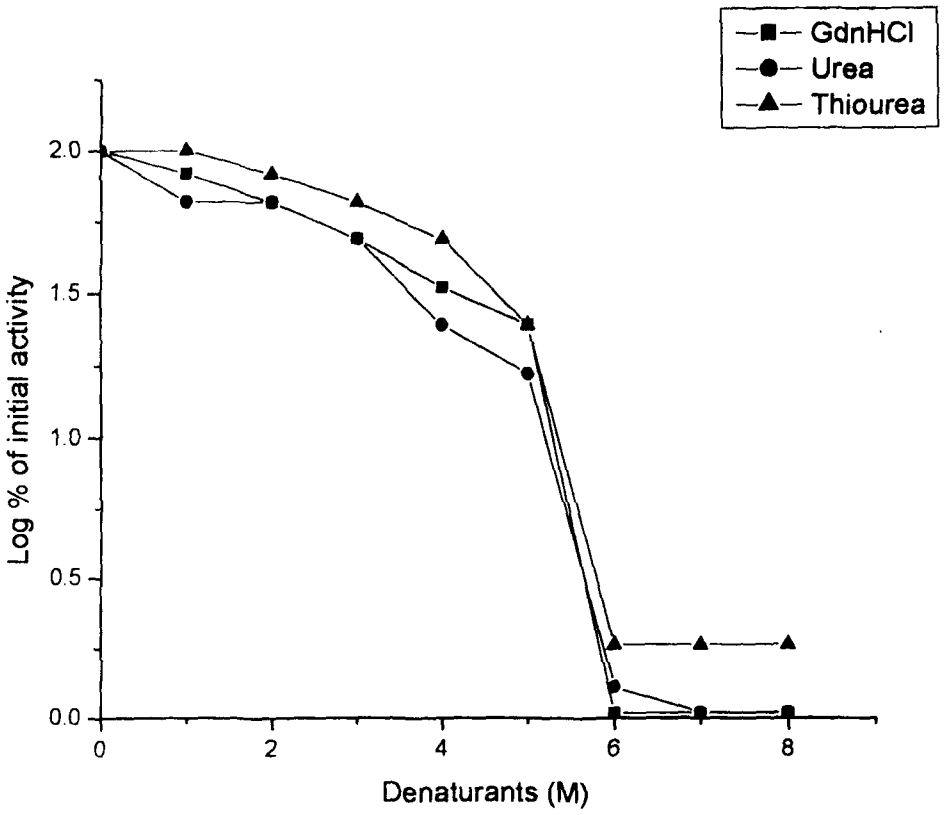


Figure 32. Effect of increasing concentration of denaturants on the activity of BBL.

BBL (125 μg/ ml) with initial titre value of 256 (100% activity) was incubated with varying concentration (0-8 M) of GdnHCl, urea and thiourea in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for four hours. The residual activity was measured by microtitre plate assay. The initial activity (100%) refers to the hemagglutinating activity of the unincubated protein.

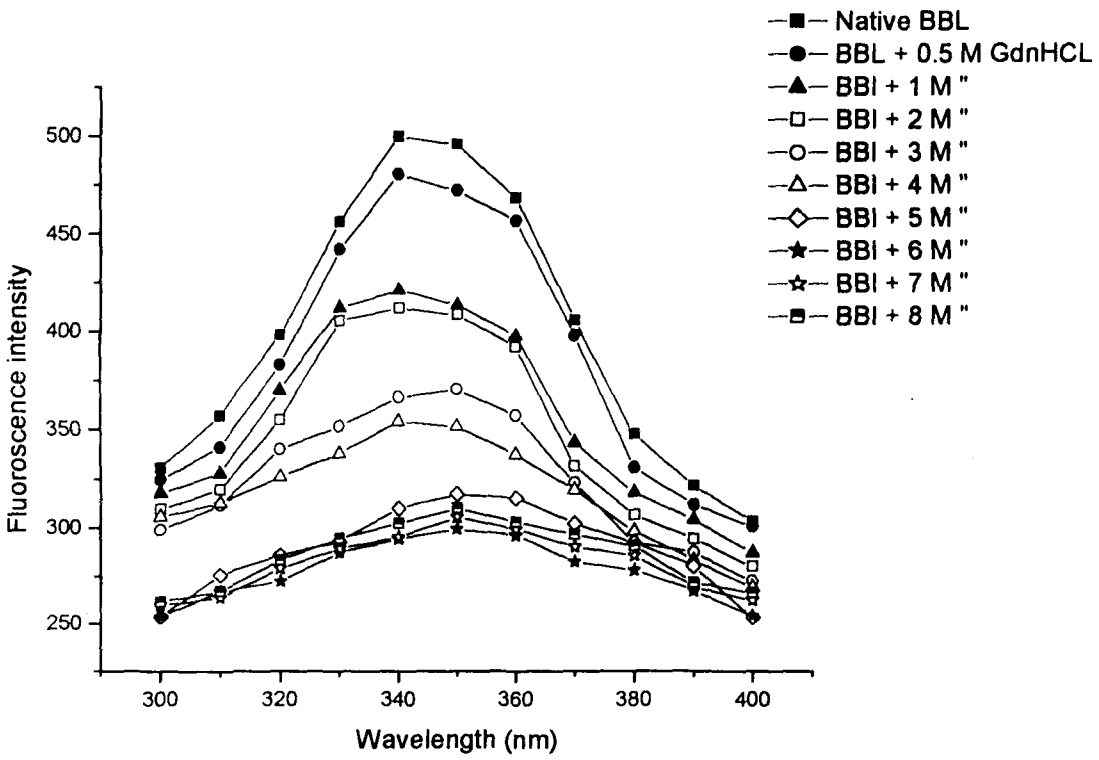


Figure 33. Effect of increasing concentration of GdnHCl on fluorescent spectra of BBL.

BBL (45μg/ml) was incubated with varying concentrations (0-8 M) of GdnHCl in 75 mM of PBS, pH 7.2 at 37°C for four hours. The emission spectra were measured in the range of 300–400 nm after excitation of protein at 280 nm.

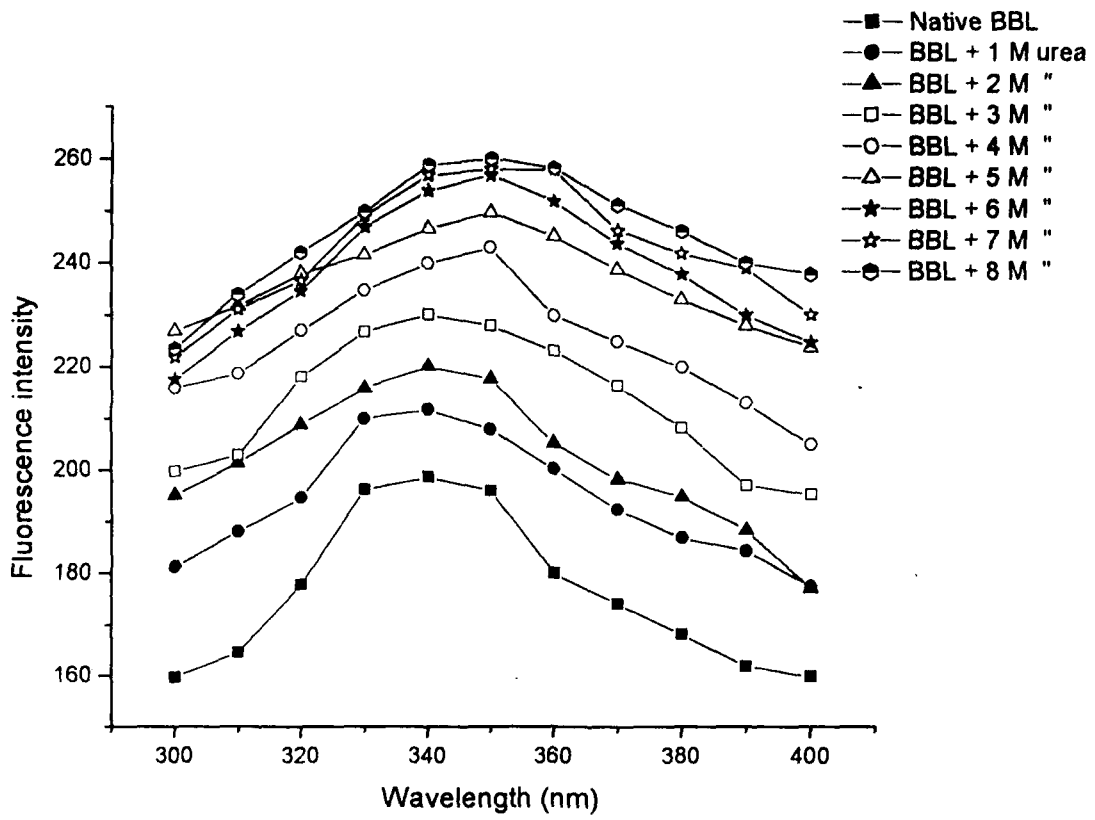


Figure 34. Effect of increasing concentration of urea on fluorescent spectra of BBL.

BBL (45μg/ml) was incubated with varying concentrations (0-8 M) of urea in 75 mM of PBS pH 7.2 at 37°C for four hours. The emission spectra were measured in the range of 300-400 nm after excitation of protein at 280 nm.

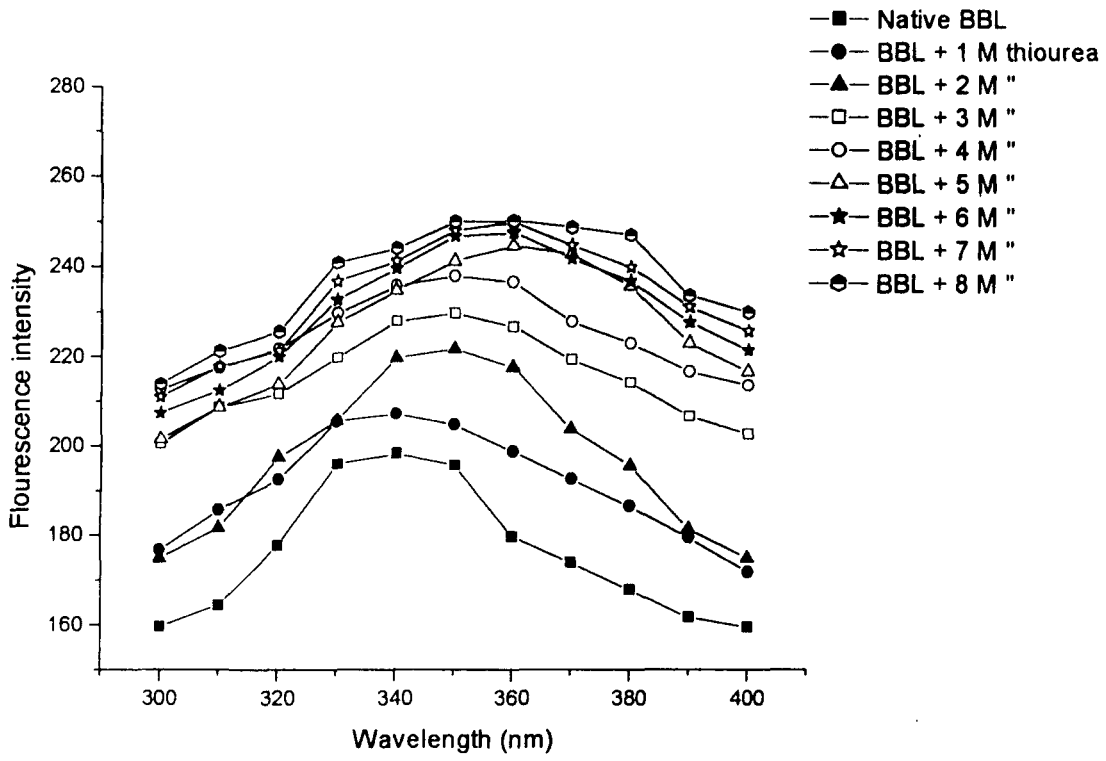


Figure 35. Effect of increasing concentration of thiourea on fluorescent spectra of BBL.

BBL (45µg/ml) was incubated with varying concentrations (0-8 M) of thiourea in 75 mM of PBS pH 7.2 at 37°C for four hours. The emission spectra were measured in the range of 300-400 nm after excitation of protein at 280 nm.

The deglycosylated lectin was eluted later than the native form of protein. The delayed elution may reflect a contraction in molecular size due to the removal of carbohydrate moiety The molecular weight and stokes radius of deglycosylated lectin corresponded to 27.7 kDa and 24 Å respectively, thus depicting a negligible change upon deglycosylation (Fig 37, 38).

Effect of temperature

The deglycosylated form retained its full activity till 40°C unlike the native form which was fully active till 45°C (Fig. 39). A sudden decrease in titre value was observed when both the forms of BBL were heated between 45° to 65°C with deglycosylated BBL displaying less stability against heat in comparison to the glycosylated BBL. Moreover, native BBL exhibited 12.5 % activity at 70° C in comparison to negligible 3.125 % of original activity of deglycosylated BBL. At 60°C, the native and deglycosylated lectin showed 25% and 12.5 % of initial activity, respectively. Glycosylated protein was significantly more stable than non-glycosylated preparation when incubated at 60°C for different time intervals. The native protein retained 12.5 % of initial activity after 1 hour of incubation at 60°C whereas the deglycosylated form exhibited 6.25 % of residual activity under similar conditions (Fig. 40).

Effect of pH

There was no difference in pH optima of glycosylated and deglycosylated lectin. Both forms exhibited similar pH optima between 7.0-7.5, with deglycosylating lectin showing less activity than the native form at pH values other than pH optima (Fig. 41).

Effect of denaturants

Effect of GdnHCl, urea and thiourea on deglycosylated forms at different concentrations has been shown in Fig. 42, 43, and 44, respectively. There was not much difference in the activity of native BBL and deglycosylated form after its incubation with 1.5 M GdnHCl for four hours. The hemagglutinating activity showed a considerable decrease from 2.5 M GdnHCl to 8.0 M GdnHCl. Native BBL showed a 50% decrease in its activity at 3.0 M GdnHCl, whereas deglycosylated form lost 75 % of its original activity at 3.0 M GdnHCl under similar conditions. Incubation with higher (6-8 M) concentrations of denaturants resulted in a complete loss in the agglutinating activity of both the forms of protein.

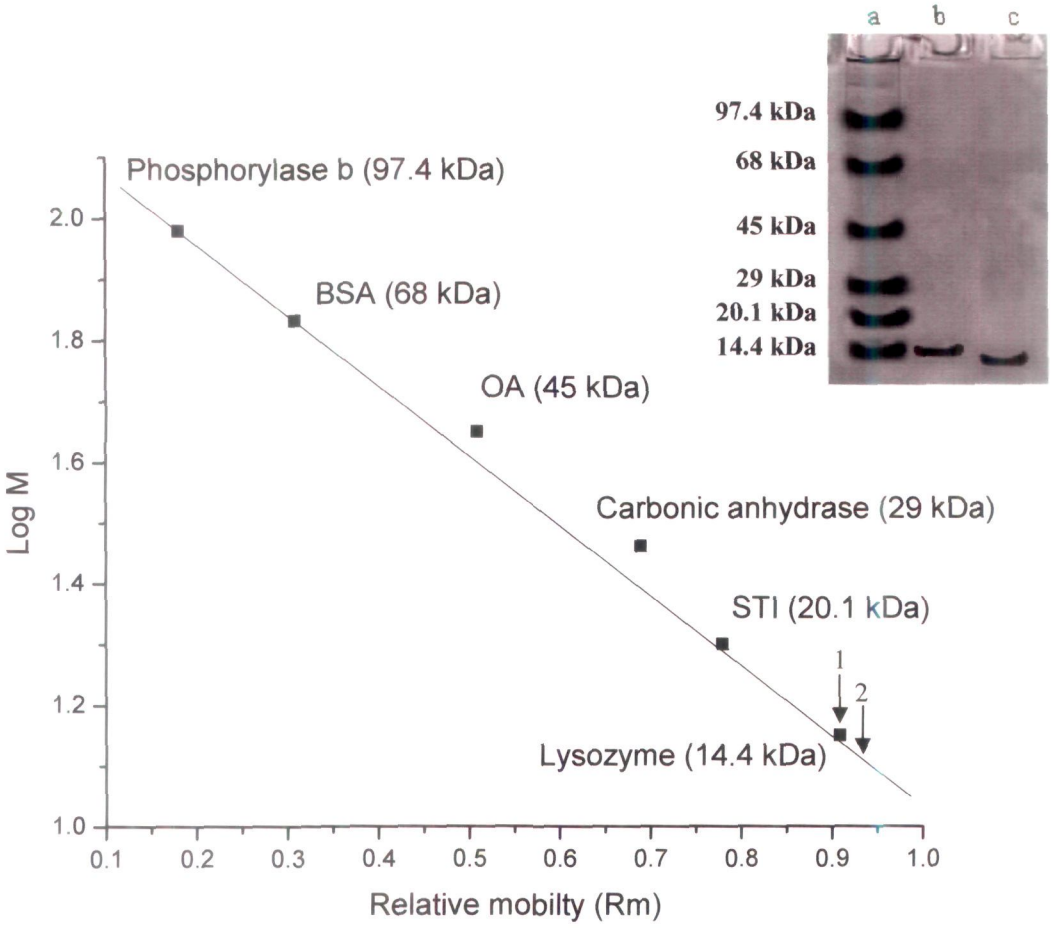


Figure 36. SDS-PAGE of native and deglycosylated forms of BBL

Electrophoresis of both native and deglycosylated BBL was performed on 12.5 % acrylamide gel in reducing condition. The relative mobilities (Rm) were plotted against their molecular weight using least square analysis of the data. Molecular weight of native and deglycosylated BBL is indicated by an arrow 1 and 2, respectively. Inset shows the electrophoretic pattern of native and deglycosylated BBL: Lane a: molecular weight standards in the descending order; Phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Soyabean trypsin inhibitor (20.1kDa), Lysozyme (14.4 kDa), Lane b: 35µg of purified native protein. Lane c: 35 µg of deglycosylated protein. Gel was stained using coomassie brilliant blue R-250 dye.

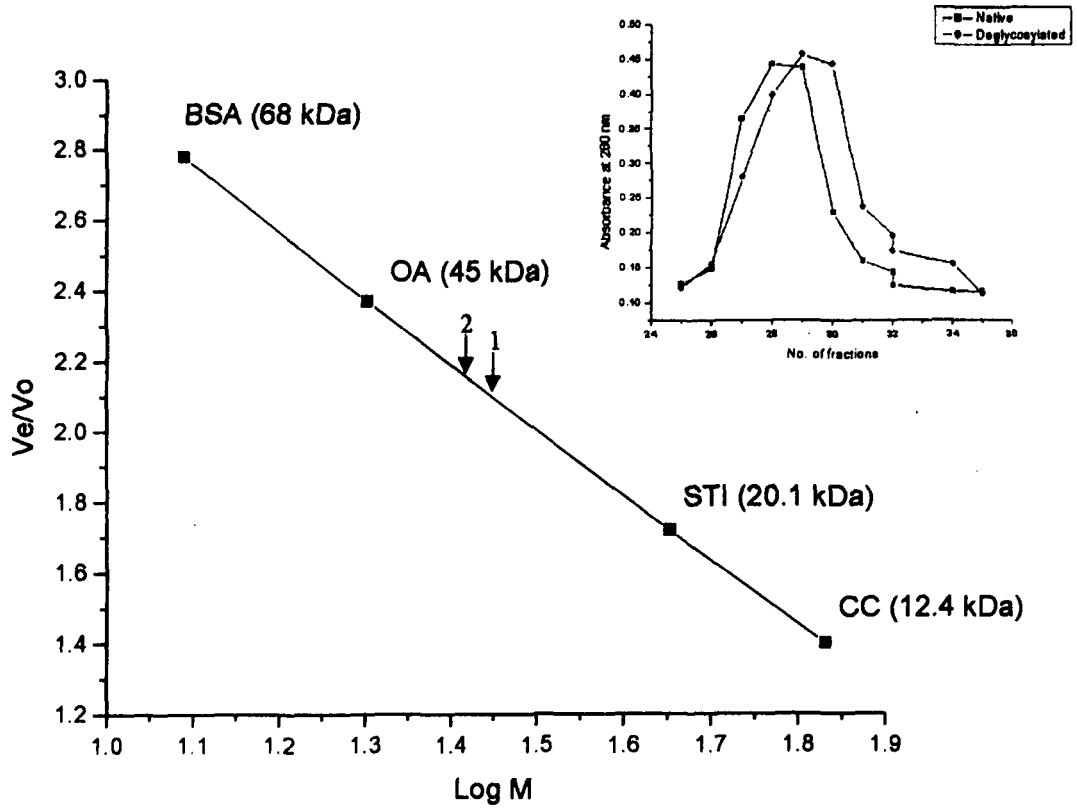


Figure 37. Molecular weight determination of deglycosylated BBL using Sephadex G-100 gel filtration chromatography.

Native and deglycosylated BBL was applied on a column of sephadex G_{-100} (1.8 × 45 cm) and eluted with 75 mM PBS containing 5 mM β-ME and 30 mM lactose, pH 7.2 at a flow rate of 15 ml/hr in the form of 1.5 ml fractions. The molecular weight markers used were bovine serum albumin (BSA, 68 kDa), ovalbumin (OA, 45 kDa), soyabean trypsin inhibitor (STI, 20.1 kDa) and cytochrome c (CC, 12.4 kDa). The elution position of the native and deglycosylated lectin is indicated by arrow 1 and 2, respectively. Inset shows the elution profile of native and deglycosylated BBL from sephadex G-100 column.

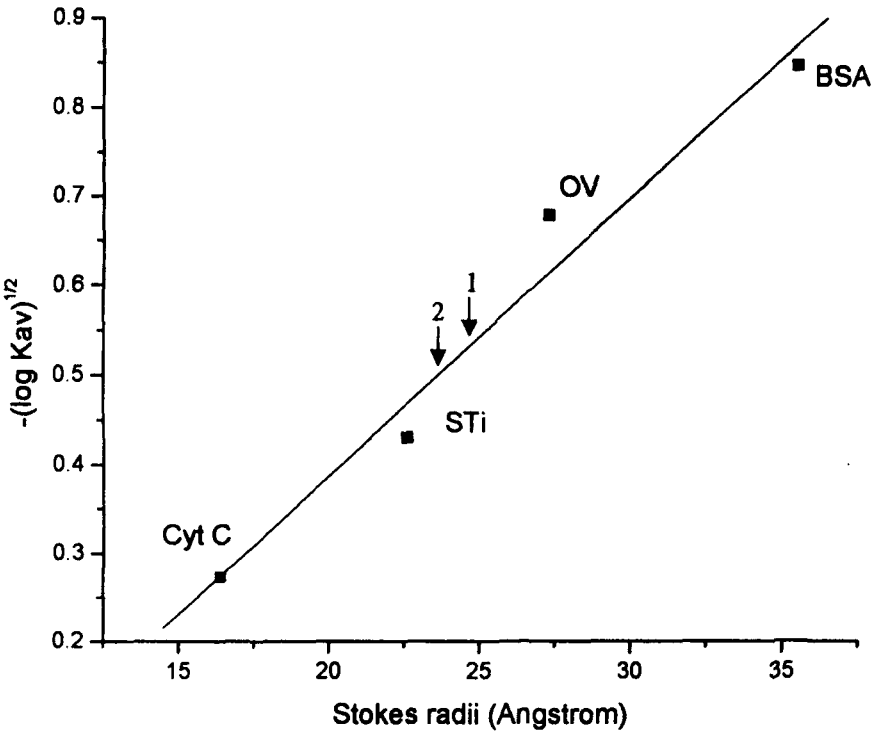


Figure 38. Determination of Stokes radius of the deglycosylated BBL by Laurent and Killander plot.

The deglycosylated lectin was subjected to gel filtration on sephadex G_{-100} column. The Kav values were computed from the elution volumes of marker proteins. Stokes radii for the marker proteins were bovine serum albumin (BSA, 35.5 Å), ovalbumin (27.3 Å), soyabean trypsin inhibitor (22.6 Å), and Cytochrome C (16.4 Å). Stokes radii of native and deglycosylated BBL is indicated by arrows 1 and 2, respectively.

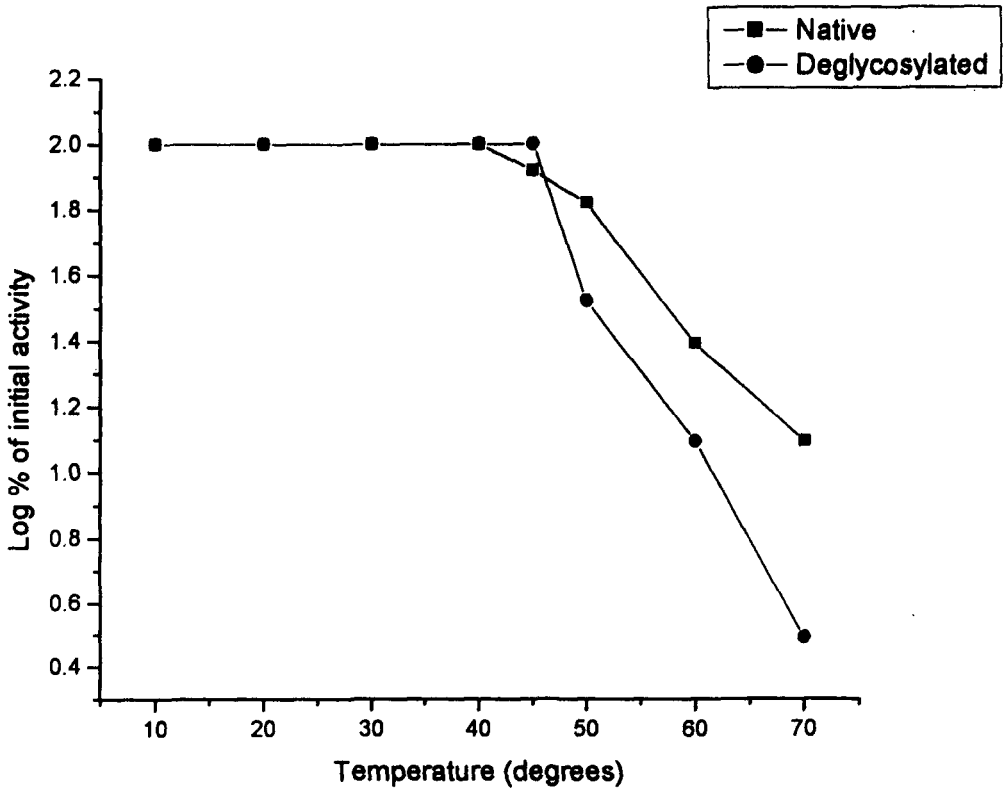


Figure 39. Effect of temperature on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) in 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl and 5 mM β-ME were incubated at various temperatures (30°C-70°C) for 30 min., chilled in crushed ice and hemagglutinating activity was assayed by microtitre plate assay. Initial activity refers to the activity of the proteins at 37°C.

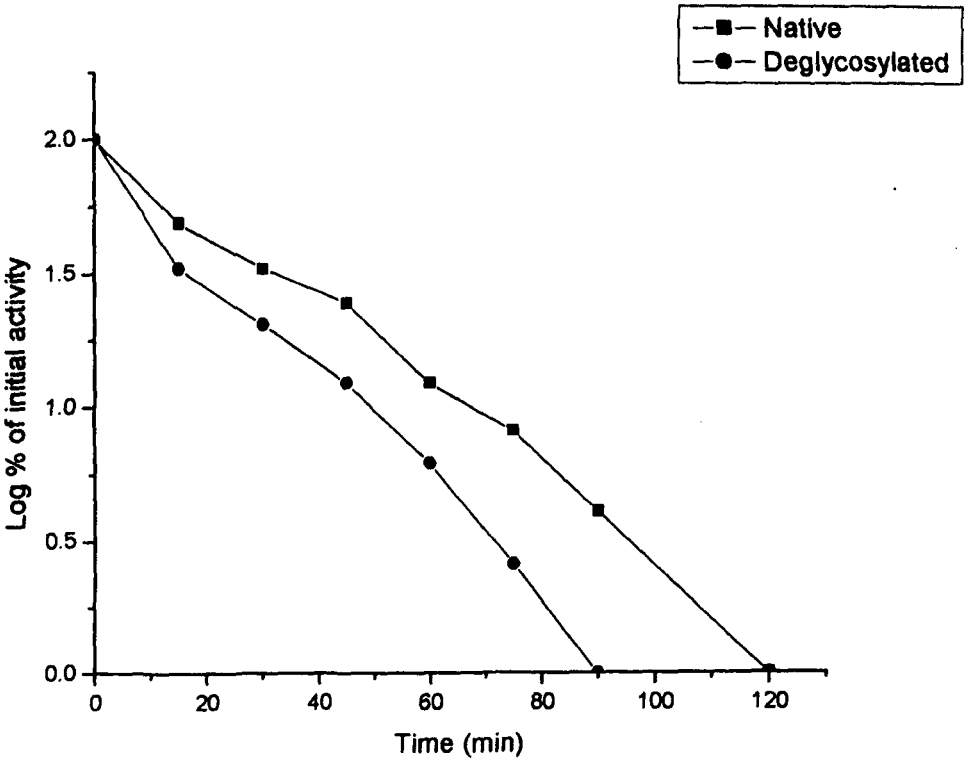


Figure 40. Thermal denaturation profile of native and deglycosylated BBL at 60° C.

Native and deglycosylated protein (125 μg/ ml) were taken in 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl and 5 mM β-ME, chilled in crushed ice and hemagglutinating activity was assayed by microtitre plate assay at 60 °C after different time intervals. Initial activity refers to the activity of the unincubated proteins.

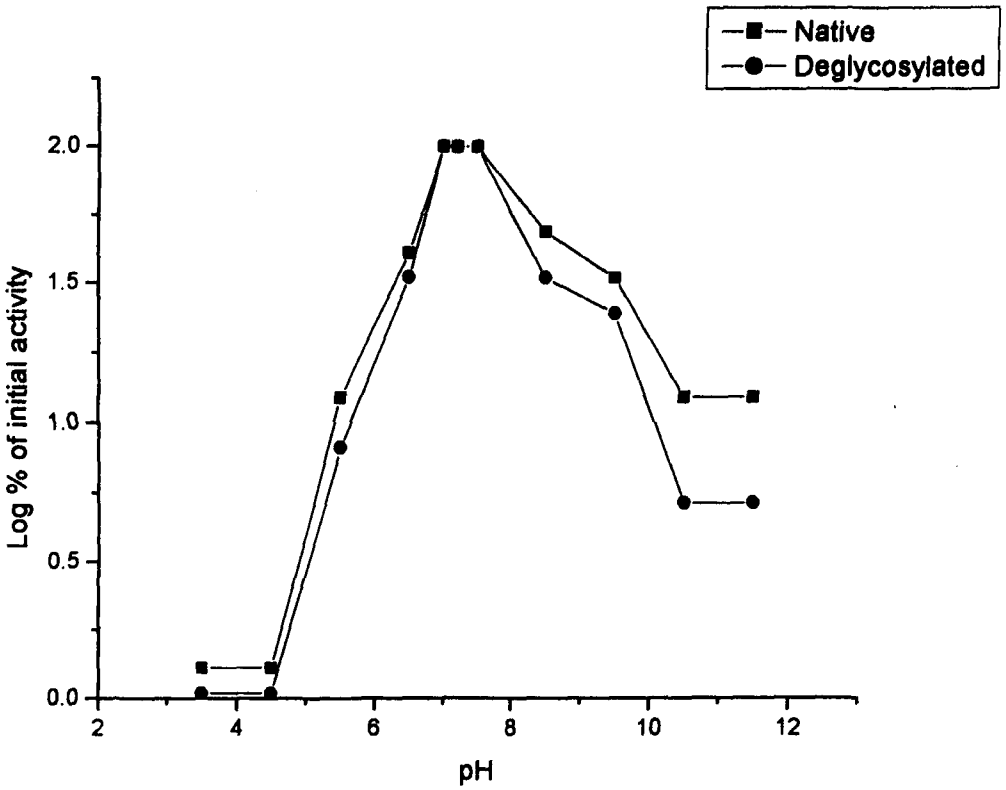


Figure 41. Effect of pH on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) were incubated in various buffers (0.1 M sodium acetate/sodium phosphate/Tris-HCl/ glycine NaOH) of different pH values (3.5-11.5) at 4°C for 24 hours. Hemagglutinating activity was assayed by microtitre plate assay. Initial activity refers to the activity of both the proteins in normal saline containing 5 mM β-ME.

Similar denaturation curve was obtained in the presence of urea, but with a pronounced decrease in case of deglycosylated form. There was a reduction of 66.66% of the initial activity of deglycosylated BBL at 3.0 M urea as compared to 50 % loss in the activity of glycosylated lectin.

Thiourea also abolished the activity of modified form but at a lower concentration than the native form.

Effects of detergents

In the presence of increasing concentration of SDS (Fig. 45) the activity of both glycosylated and deglycosylated lectins showed a decrease with a pronounced trend for deglycosylated form.

Similarly, pre-incubation of both forms of lectin with Tween-20 at 37°C for 1 hour resulted in a greater loss of activity (Fig. 46) for deglycosylated BBL than for the glycosylated lectin. Native lectin retained 50 % of its initial activity at a concentration of 5 % (v/v) of Tween-20, whereas the deglycosylated preparation lost nearly 75% of activity under similar concentration and conditions. The effect of increasing concentration of Triton X-100 (0.5-5.0 %, (v/v)) is illustrated in Fig. 47. The hemagglutinating activity of native and deglycosylated form was decreased at all concentrations, with native protein retaining higher activity as compared to the deglycosylated form. At 5.0 % (v/v) concentration of Triton X-100, native exhibited 66.6 % of the initial activity, whereas only 33.3 % of initial activity was retained by deglycosylated form.

Spectrophotometric properties

UV spectra of both deglycosylated and native lectins showed maxima at 282 nm (Fig. 48), with slightly higher absorbance for deglycosylated BBL. Moreover, fluorescent spectra for deglycosylated form displayed a higher fluorescent intensity profile (Fig. 49) with similar maximum emission wavelength at 335 nm as that for native protein. Similarly, circular diochroism spectra of deglycosylated BBL did not show any appreciable change with respect to the spectra of native BBL, thus suggesting no alteration in the secondary structure of lectin upon removal of sugar moiety (Fig. 50).

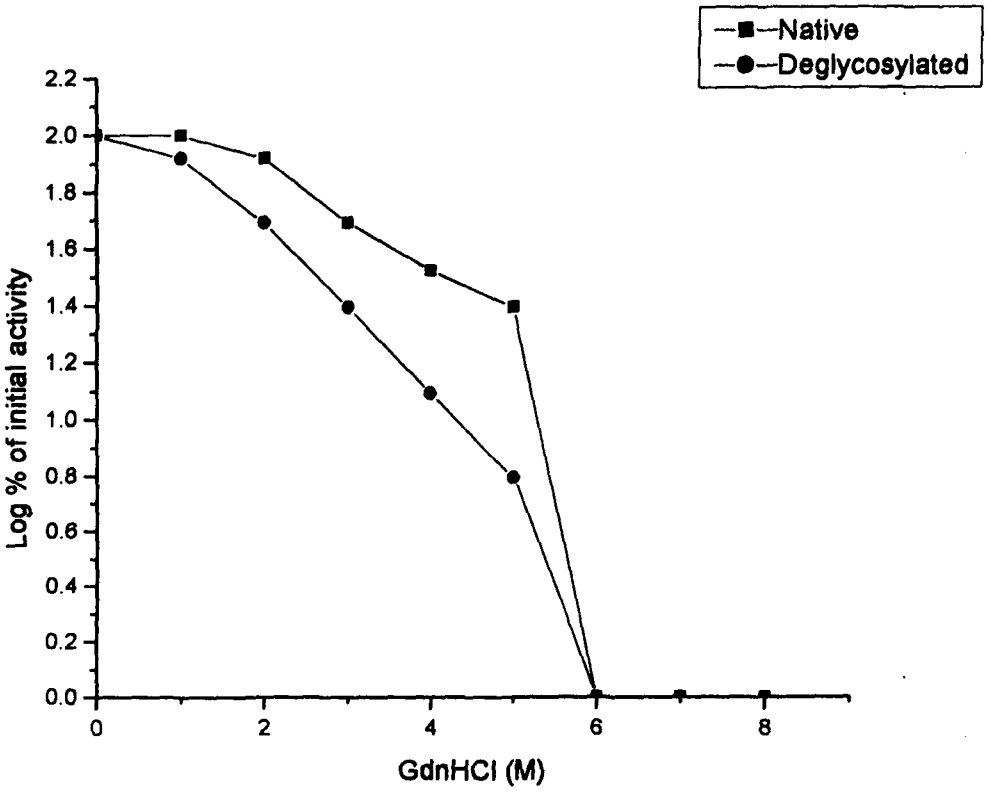


Figure 42. Effect of increasing concentration of GdnHCl on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) were incubated in various concentration of GdnHCl (0-8 M) in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for four hours. Hemagglutinating activity was assayed using microtitre plate assay and the initial activity refers to the activity of unincubated protein.

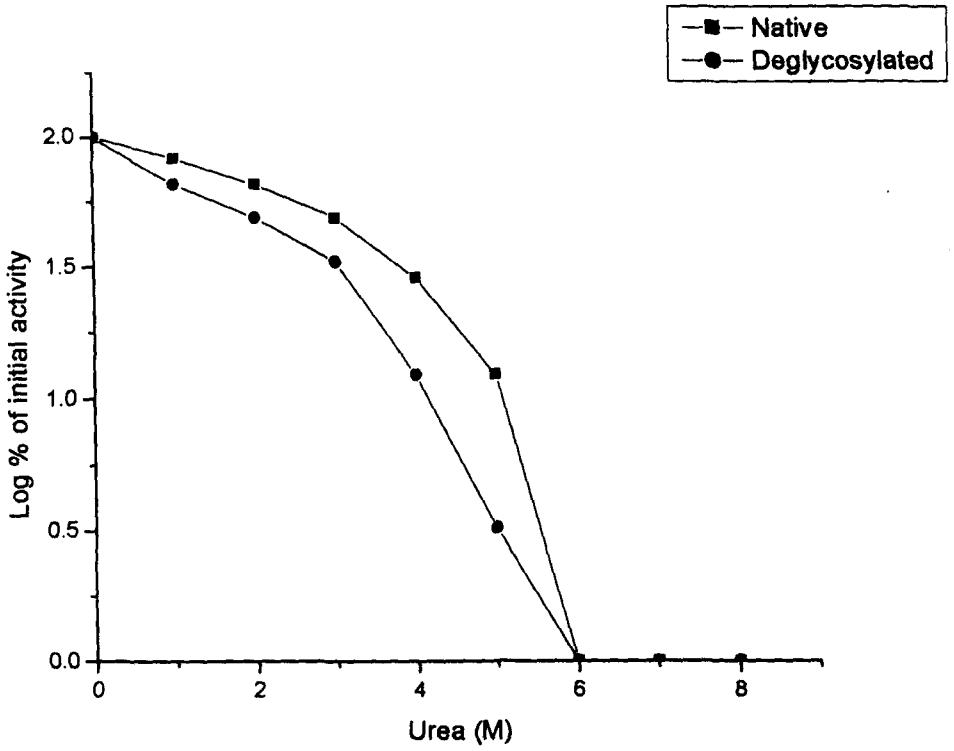


Figure 43. Effect of increasing concentration of urea on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) were incubated in various concentration of urea (0-8 M) in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for four hours. Hemagglutinating activity was assayed using microtitre plate assay; the initial activity refers to the activity of unincubated protein.

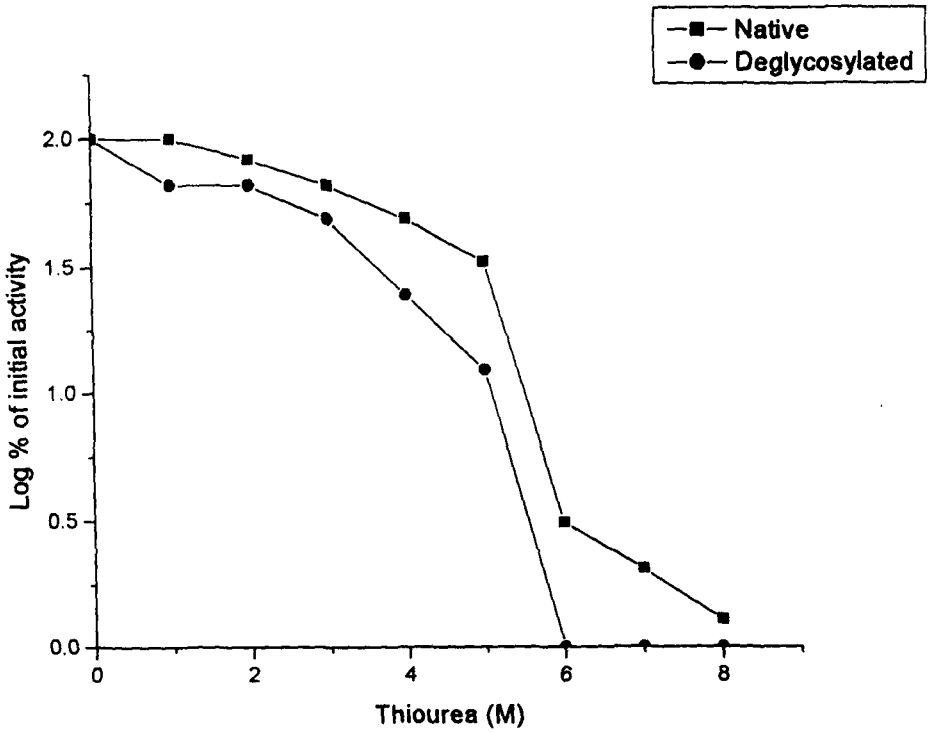


Figure 44. Effect of increasing concentration of thiourea on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) were incubated in various concentration of thiourea (0-8 M) in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for four hours. Hemagglutinating activity was assayed using microtitre plate assay and the initial activity refers to the activity of unincubated protein.

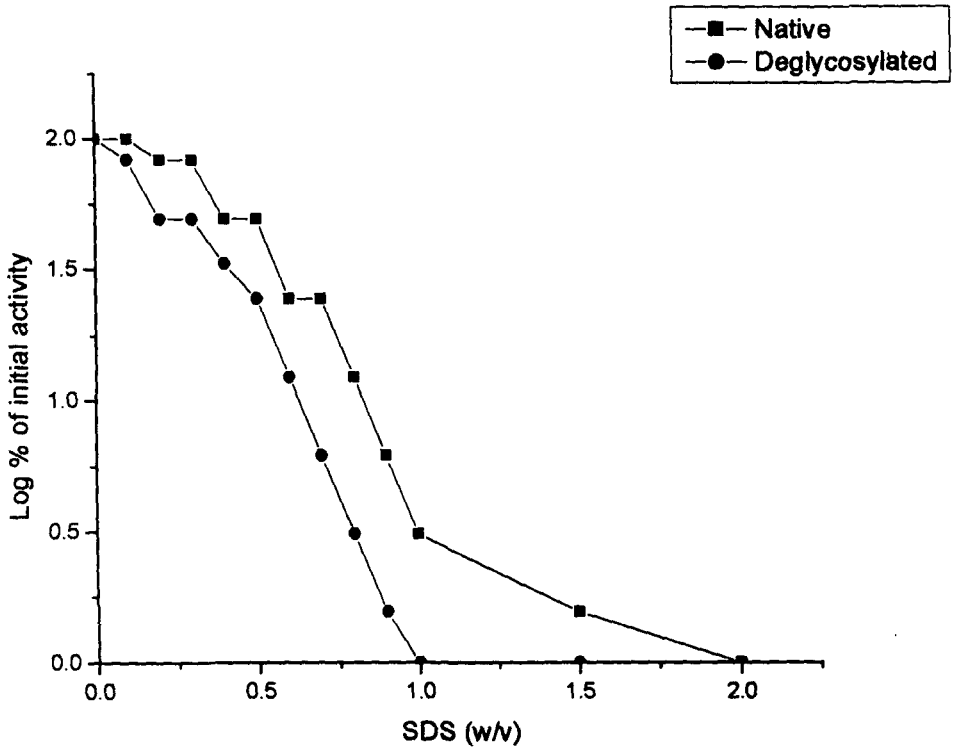


Figure 45. Effect of increasing concentration of SDS on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) were incubated at various concentration of SDS (0.1-2 mg/ml) in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for one hour. Hemagglutinating activity was assayed using microtitre plate assay and the initial activity refers to the activity of unincubated protein.

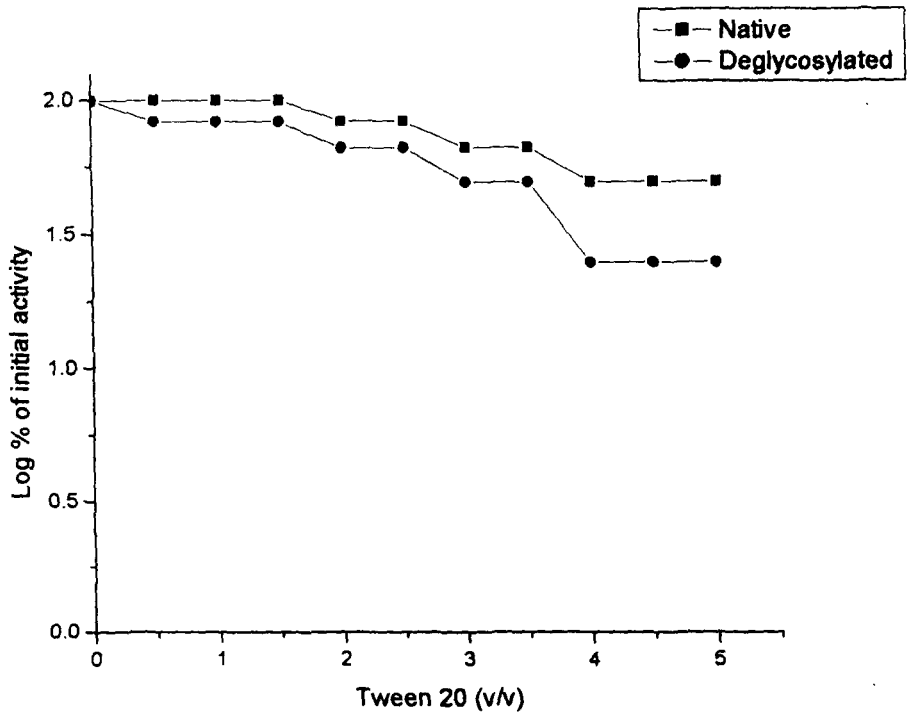


Figure 46. Effect of increasing concentration of Tween-20 on native and deglycosylated BBL.

Native and deglycosylated protein (125 µg/ ml) were incubated at various concentration of Tween-20 (0.5-5 %) in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for one hour. Hemagglutinating activity was assayed using microtitre plate assay and the initial activity refers to the activity of unincubated protein.

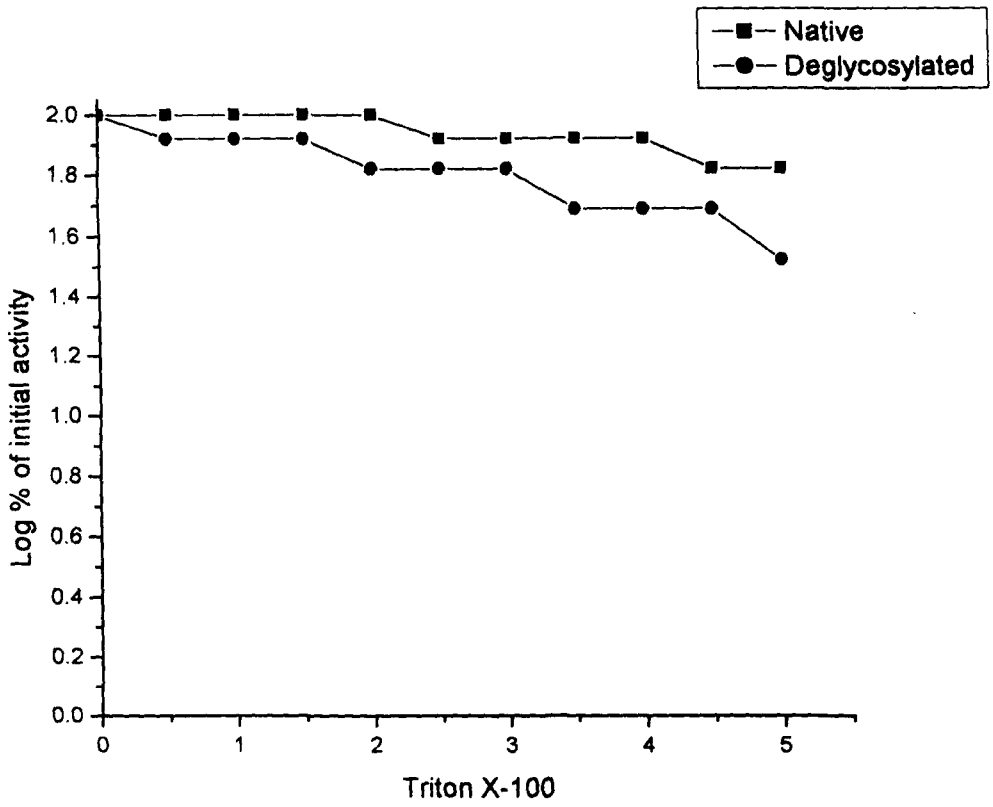


Figure 47. Effect of increasing concentration of Triton X-100 on native and deglycosylated BBL.

Native and deglycosylated protein (125 μg/ ml) were incubated at various concentration of Triton X-100 (0.5-5%) in 75 mM of PBS, pH 7.2 containing 5 mM β-ME at 37°C for one hour. Hemagglutinating activity was assayed using microtitre plate assay and the initial activity refers to the activity of unincubated protein.

Fluorescence studies of Denaturant treated native and deglycosylated form

Fig. 51 demonstrates the unfolding pathway of glycosylated and deglycosylated BBL in the presence of increasing concentration of GdnHCl. There was a gradual decrease in the fluorescence intensity of both forms of lectin till 6 M GdnHCl with a pronounced red shift from 335 to 357 nm (for BBL). A slight increase in the intensity was observed at higher concentrations with no change in the emission maxima. However, in case of deglycosylated BBL, higher fluorescence intensity was observed than the native lectin with a greater red shift at all concentration of GdnHCl.

Fig. 52 and 53 depicts the fluorescent profile for both the forms of lectin at different concentrations of urea and thiourea, respectively. Complete unfolding of protein was observed at 8 M urea with an increase in fluorescence intensity and shift of emission maximum from 335 to 355 nm. Thiourea caused a similar increase in the fluorescence intensity with a red shift from 335 nm to 360 nm. The deglycosylated buffalo brain lectin in the presence of urea and thiourea, again exhibited higher fluorescence intensity with a greater red shift as compared to the glycosylated native lectin. The difference in the fluorescence intensity of native and deglycosylated form was less between 1 to 2 M concentrations of denaturants, but became significantly higher at higher concentration of urea and thiourea.

Immunological studies

Evalution of antibody response

Lectin obtained from buffalo brain was highly immunogenic in rabbits as it readily gave a single line of identity when tested by double immunodiffusion indicating the homogeneity of protein preparation. Direct binding ELISA was performed to characterize the immune response in rabbits using pure buffalo brain lectin as antigen. The antiserum showed a high titre >128000 (Figure 54) suggesting the high immunogenic nature of BBL. Preimmunized serum served as negative control and did not show any appreciable binding to buffalo brain lectin.

Antigenic cross reactivity with other brain lectins

Immunodiffusion studies show that anti-BBL antibodies recognized both goat and sheep brain lectins, but comparatively faint precipitin lines were observed with respect to buffalo brain lectin indicating that antibody was not highly specific to sheep and goat lectin as compared to buffalo lectin (Fig. 55). The specificity of the induced antibodies and sharing of common antigenic determinants between lectins

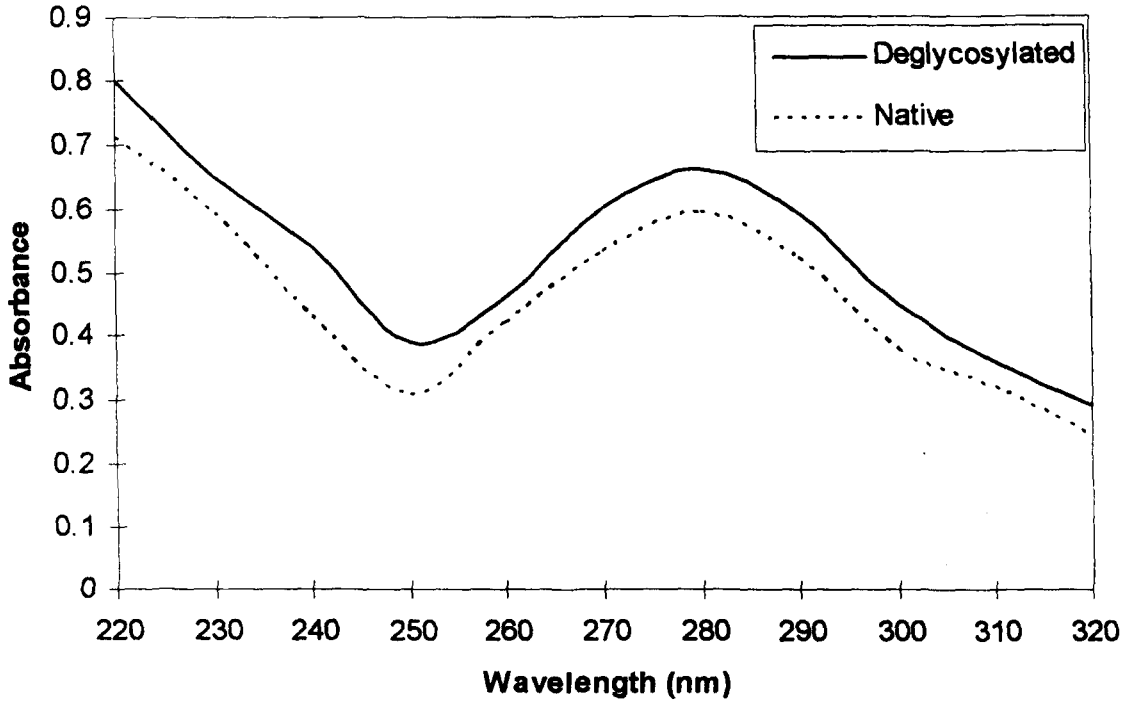


Figure 48. Ultra violet spectra of native and deglycosylated BBL.

Native and deglycosylated BBL (150μg/ml) were prepared in 75 mM PBS, pH 7.2 containing 5 mM β-ME. The spectra were recorded between wavelength 220-320 nm.

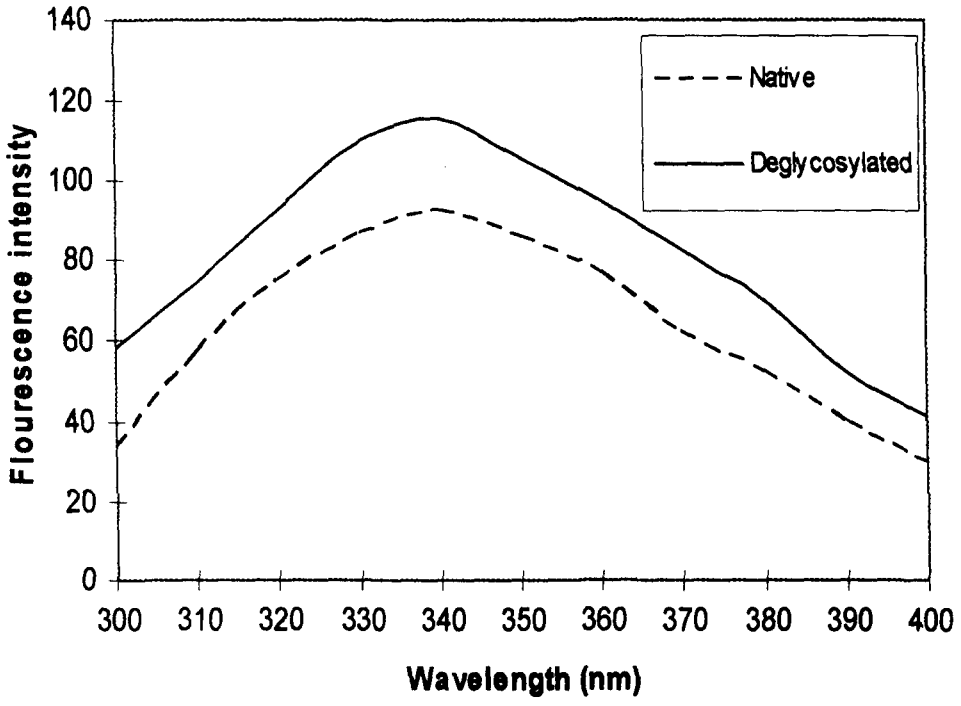


Figure 49. Intrinsic fluorescence of native and deglycosylated BBL.

Native and deglycosylated BBL (45μg/ml) were prepared in 75 mM PBS, pH 7.2. The intrinsic fluorescence spectra were recorded by exciting the protein at 280 nm and the emission wavelength were taken between 300-400 nm.

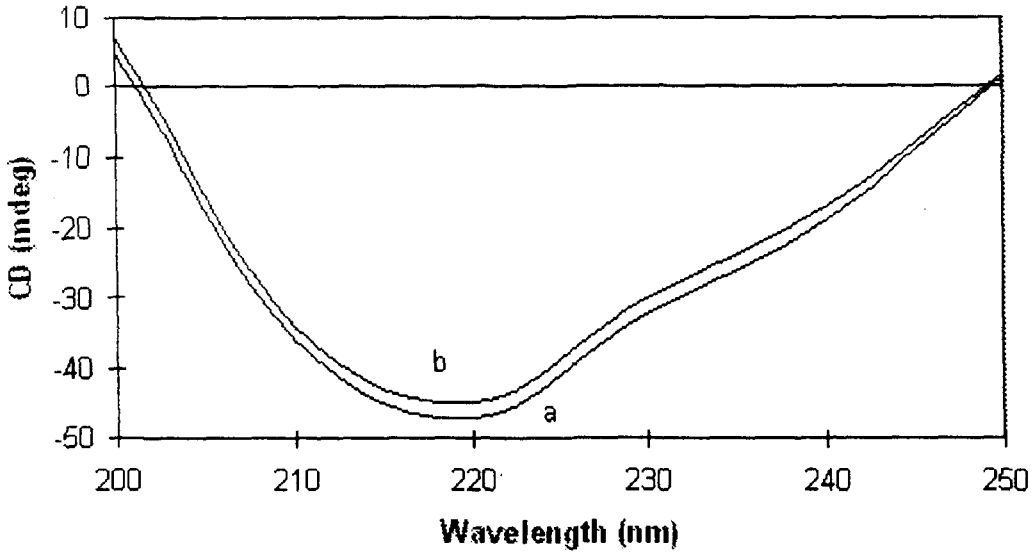


Figure 50. Far- UV-CD spectra of native and deglycosylated BBL

The spectra of native BBL (a) and deglycosylated BBL (b) (0.250 mg/ml) in 10 mM PBS pH 7.2 were recorded between 200-250 nm using path length of 0.1 cm.

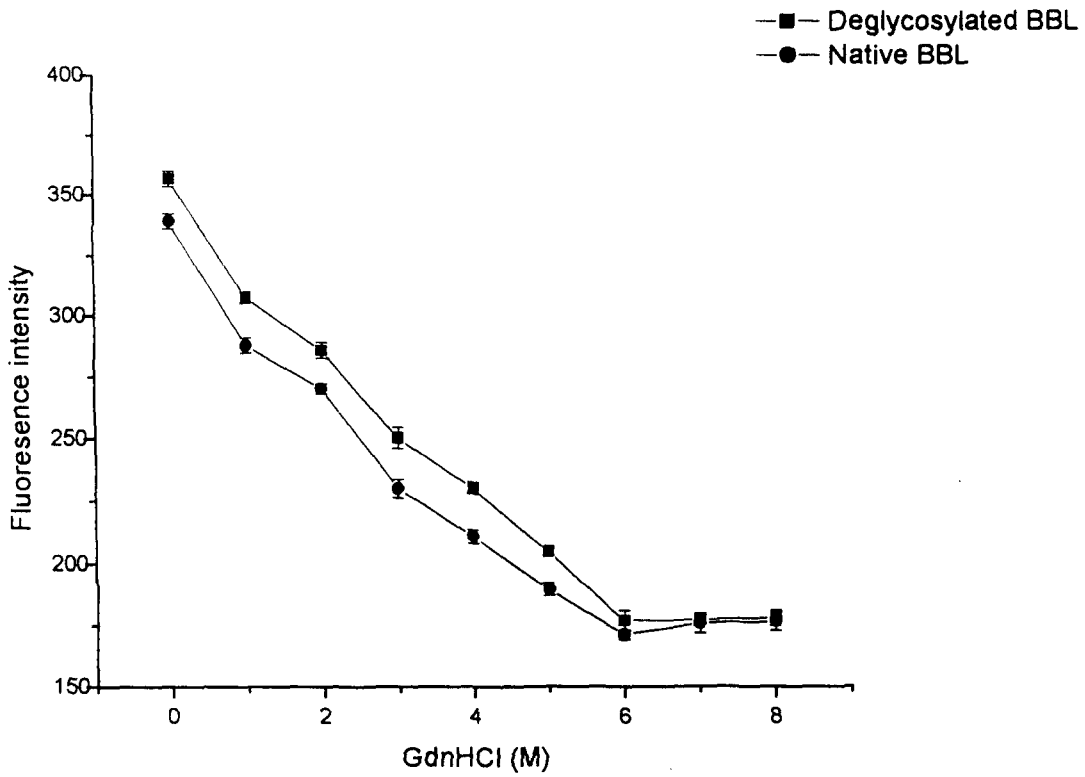


Figure 51. Effect of increasing concentration of GdnHCl on fluorescence intensity of glycosylated and deglycosylated forms of BBL.

Native and deglycosylated BBL (40µg/ ml) were incubated in 0-8 M concentration of GdnHCl 75 mM of PBS pH 7.2 at 37°C for four hours. The proteins were excited at 280 nm and emission wavelengths were taken between 300-400 nm.

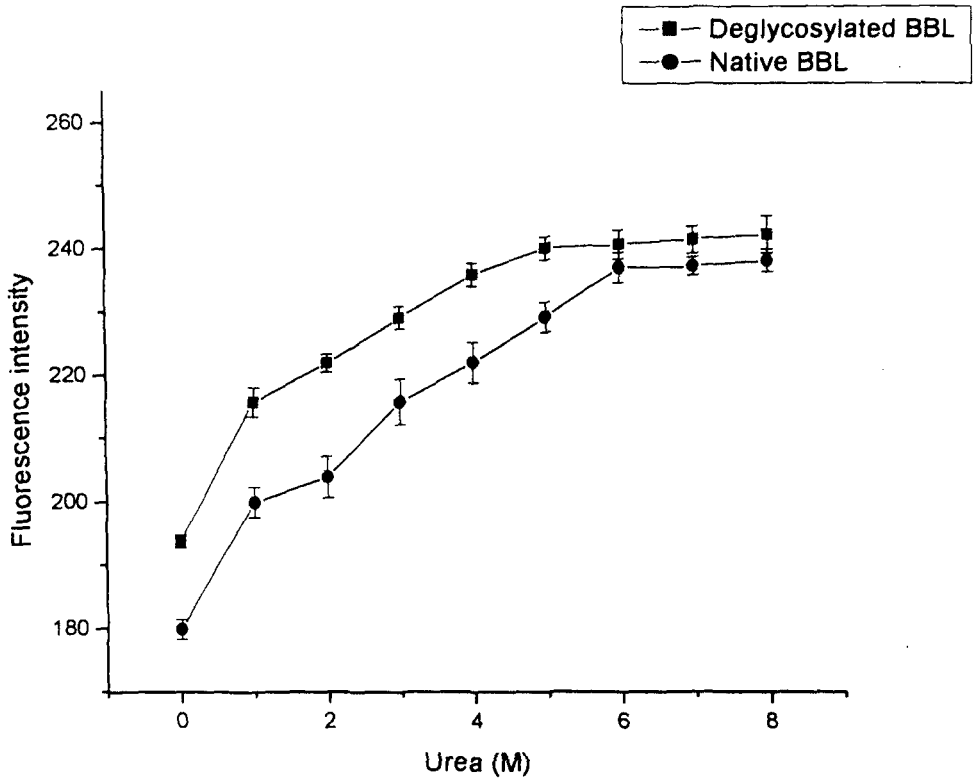


Figure 52. Effect of increasing concentrations of urea on fluorescence intensity of glycosylated and deglycosylated forms of BBL.

Native and deglycosylated proteins (40μg/ ml) were incubated in 0-8 M concentration of urea in 75 mM of PBS pH 7.2 at 37°C for four hours. The proteins were excited at 280 nm and emission wavelengths were recorded between 300-400 nm.

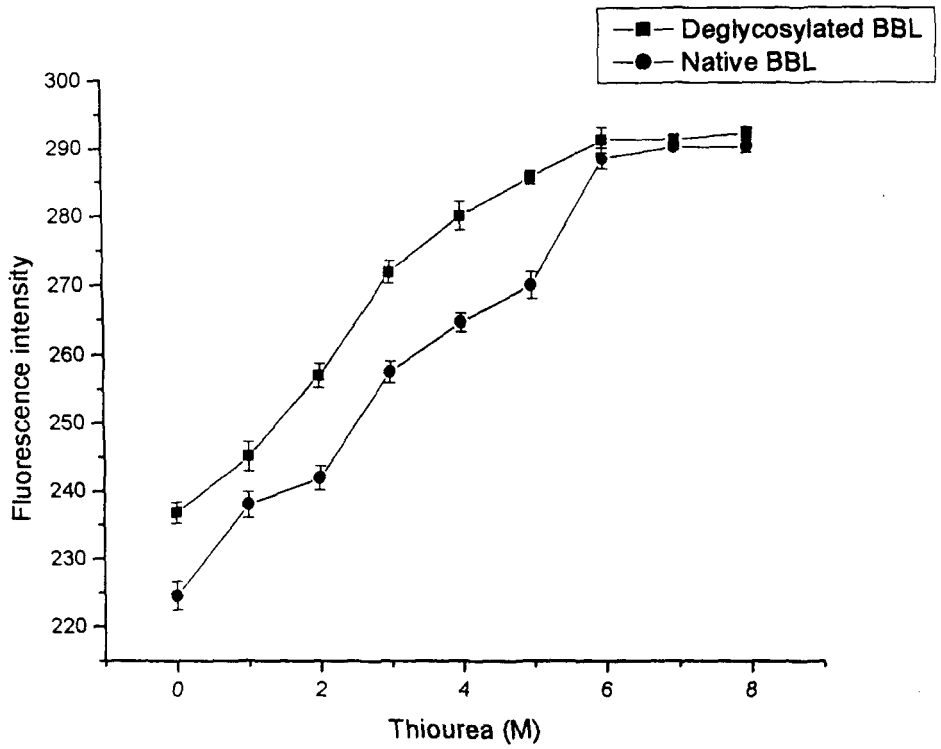


Figure 53. Effect of increasing concentrations of thiourea on fluorescence intensity of glycosylated and deglycosylated forms of BBL.

Native and deglycosylated proteins (40μg/ ml) were incubated in 0-8 M concentration of thiourea in 75 mM of PBS, pH 7.2 at 37°C for four hours. The proteins were excited at 280 nm and emission wavelengths were taken between 300-400 nm.

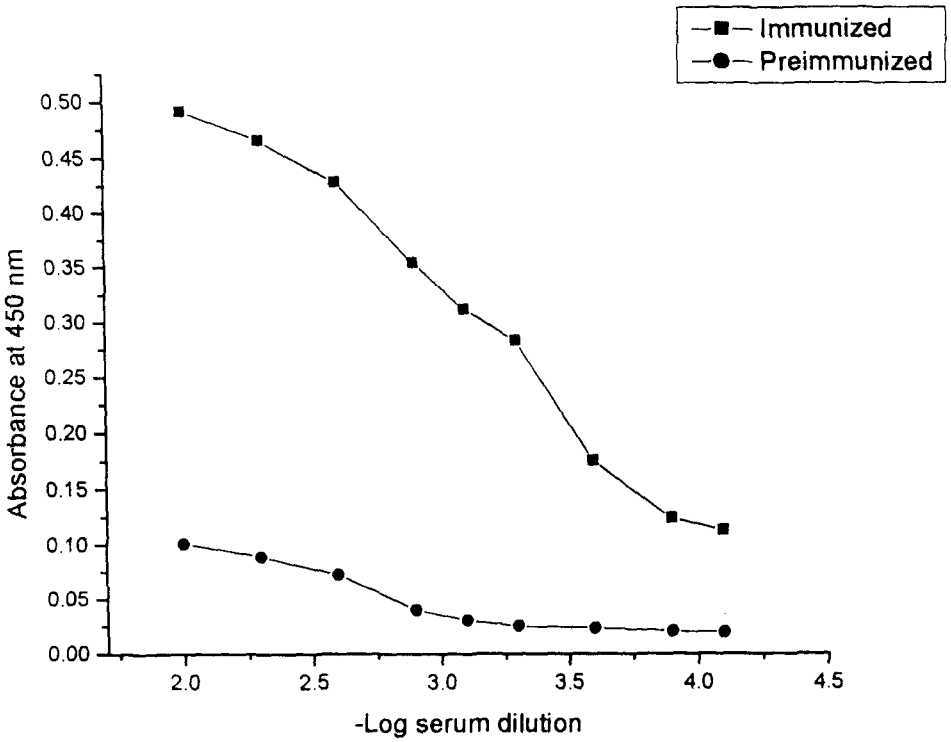


Figure 54. ELISA of anti buffalo brain lectin.

Pre-immunized and BBL immunized rabbit anti-sera tested by direct binding ELISA at different dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800). The wells were coated with a solution containing 20 μg/ml buffalo brain lectin. The negative logarithm of serum dilution versus the absorbance at 450 nm for both pre-immunized and immunized sera are plotted.

was further ascertained by competition ELISA, using purified sheep, goat, and buffalo brain lectins as competitors. The induced antibodies showed a high degree of specificity for buffalo brain lectin (immunogen), however, the extent of binding was slightly slightly lower for sheep and goat lectins. The buffalo brain lectin inhibited the anti buffalo antibody binding to solid phase bound antigen by 87%, while the inhibition of goat and sheep lectin was 53% and 62%, respectively (Fig. 56) The antibodies were quite specific for the immunogen, since only 5 μg/ml of BBL was required for achieving 50% inhibition. (Table VII). Anti buffalo lectin antibody displayed full cross reactivity with goat and sheep brain lectin with concentrations required to achieve 50% inhibition corresponded to 18 and 9.5 μg/ml, respectively and the relative affinities for sheep and goat lectins were found to be 60.91% and 71.26% respectively (Table VII).

The antiserum was also tested against various tissue homogenates like heart, liver and lung by dot blot analysis (Fig. 57). It was found that antibodies reacted strongly with all of them.

Membrane studies

Effect of BBL mediated agglutination on the RBC osmofragility

Extent of hemolysis of erythrocytes in the presence and absence of buffalo brain lectin is depicted in Fig. 58. Erythrocytes exhibited a decrease in hemolysis when exhibited to a range of hypotonic to isotonic NaCl solutions. Lectin agglutinated cells displayed a significant ($P<0.001$) lysis at 0.55 % and 0.65% NaCl concentration with 72.22 and 70.03% hemolysis respectively, depicting a 47.7 and 112.53 % increase with respect to lysis in untreated RBCs. In the absence of BBL, there was only 48.89 and 32.95% hemolysis at similar concentration of hypotonic solution. A significant ($P< 0.001$) 41.77% hemolysis of RBCs is observed at isotonic NaCl concentration showing pronounced lectin binding to RBC membrane.

Effect of increasing concentration of buffalo brain lectin on hemolysis of erythrocytes

The fragility of RBCs in the presence of increasing concentration of brain lectin in 75 mM PBS pH 7.4 at 37°C is shown in Fig. 59.There was a concentration dependent rise in the hemolysis of erythrocytes with maximum lysis corresponding to 81.25 % at 100 μg of lectin concentration, while negligible hemolysis was observed in control

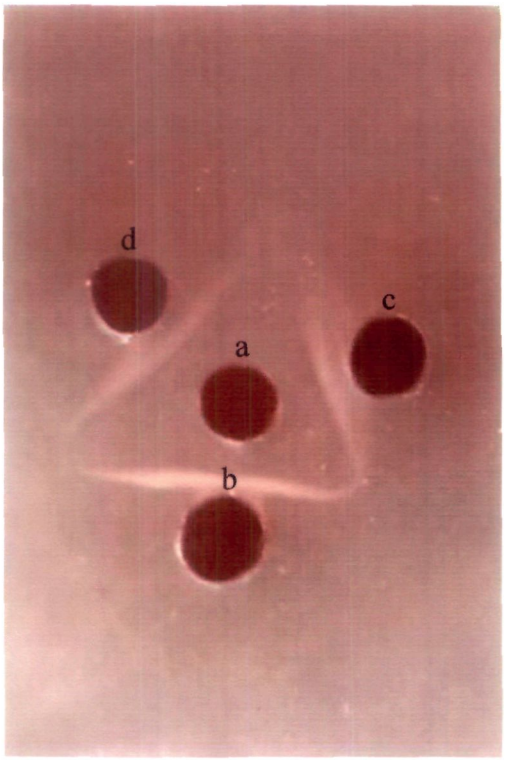


Figure 55. Comparison of antigenecity of lectin preparation from goat, sheep and buffalo brain by Ouchterlony immunodiffusion.

Well 'a' contains rabbit antiserum raised against buffalo brain lectin. Wells 'b', 'c', 'd' contain lectin (250μg/ml) from buffalo, sheep and goat brain, respectively.

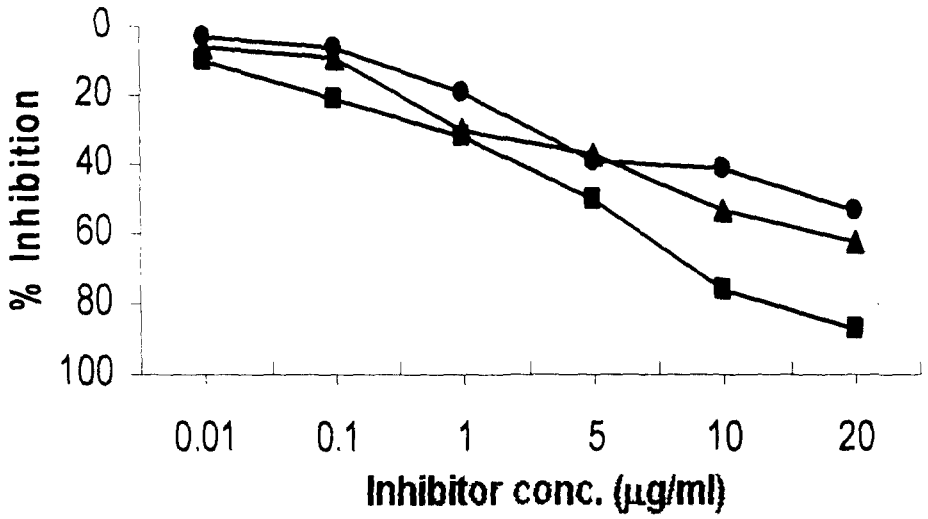


Figure 56. Competitive ELISA of anti buffalo brain lectin antibody by different lectins as inhibitors

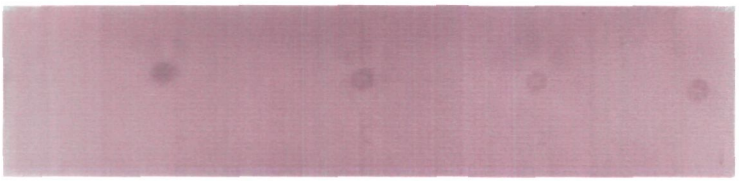
For the antigenic specificity inhibition studies, varying amounts (0-20 µg/ml) of inhibitors like purified buffalo brain lectin (■), sheep brain lectin (▲), goat brain lectin (●) were taken with a constant amount of antiserum (1:100), raised against BBL as described in 'methods' section.

Table VII.

Relative binding affinity of anti-buffalo antibodies to various brain lectins

Inhibitor	Conc. for 50% inhibition (µg/ml)	Maximum % inhibition at 20 µg/ml	% Relative Affinity
Buffalo brain lectin	5	87	100
Goat brain lectin	18	53	60.91
Sheep brain lectin	9.5	62	71.26

Each value represents the mean of three independent experiments performed in triplicates.



a b c d e

Figure 57. Dot Blot analysis using anti buffalo lectin antiserum.

Tissue homogenates from buffalo brain, heart, lung and liver were loaded on nitrocellulose membrane sheet which was stained with rabbit antiserum raised against buffalo brain lectin (1:50 dilution). The spots a, b, c, d and e correspond to control (75 mM PBS), brain, lung, heart and liver tissue homogenates (2 μg), respectively.

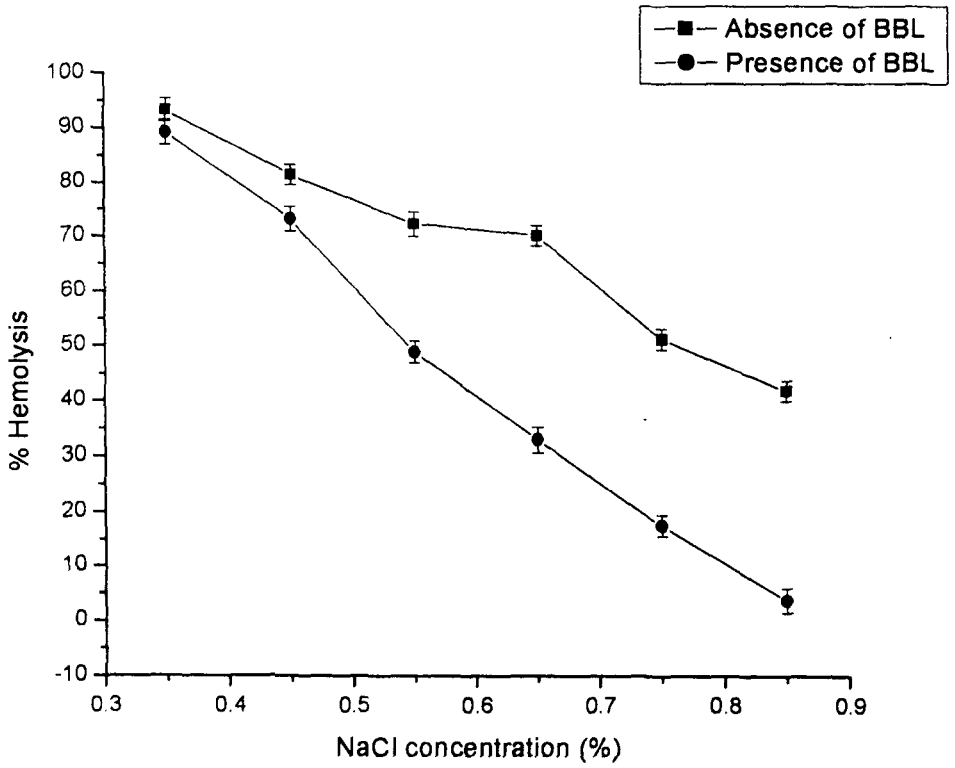


Figure 58. Effect of BBL on hemolysis of trypsinized erythrocytes at varying concentration of hypotonic solution.

Trypsinized rabbit erythrocytes at different NaCl concentrations in the absence and presence of BBL (50 μg/ml) after 4 hours of incubation. Values shown are the mean ± S.D obtained from three observations.

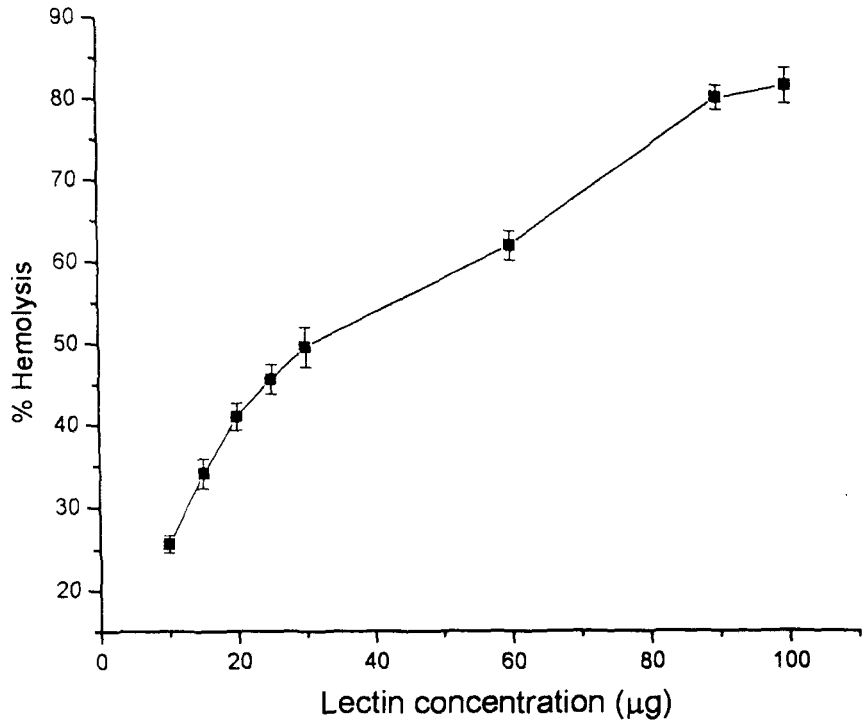


Figure 59. Effect of different concentration of BBL on hemolysis of trypsinized rabbit erythrocytes.

The incubation of 10 % RBC suspensions with different concentration of lectin (10μg-100μg/ml) was carried out for 4 hours at 37^0 C. Samples were centrifuged and RBC lysates were analysed at 540 nm. Values shown are the mean ± S.D obtained from three observations.

Effect of temperature, pH and incubation time on the lectin mediated hemolysis of RBCs

The lectin-induced hemolysis at increasing temperature is depicted in Fig. 60. A temperature dependent gradual increase in percent hemolysis was observed, with maximum and significant ($P < 0.001$) hemolysis of 83.79% at 40°C in BBL treated erythrocytes as compared to negligible hemolysis in unagglutinated cells. Preincubation of erythrocytes with BBL in various buffers of pH range (3.5-9.5) revealed a sharp enhancement of percent hemolysis with a maximum of 80.19 % at pH 7.5 (Fig. 61).

The extent of hemolysis increased with incubation period showing maximum lysis of 83.84% for BBL treated RBCs, while only 1.633% for unagglutinated erythrocytes after a time lapse of 4 hours (Fig. 62).

Effect of osmotic colloids on the hemolysis of erythrocytes in the presence and absence of brain lectin

The ability of non-electrolytes to protect lectin-induced lysis of red blood cells was assessed after incubation with different sugars in 75 mM PBS pH 7.4 for 4 hours. Lectin agglutinated cells exhibited about 72.65 % hemolysis ($P< 0.001$) in PBS (Fig. 63) as compared to 2.33 percent hemolysis of unagglutinated cells. The presence of 30 mM lactose and sucrose provided the maximum protection, with hemolysis of about 10.75 and 16.11 % respectively, while addition of galactose (39.47 % hemolyis) and glucose (60.96 % hemolysis) did not inhibit lysis. The presence and absence of colloidal osmotic media did not show any appreciable effect on hemolysis of unagglutinated cells.

Effect of brain lectin on susceptibility of erythrocytes membrane to free radical damage by pyragallol

When RBC treated with lectin for 1 and 4 hour were exposed to oxyradical shock, a release of 26.13 and 33.39 μM of oxyhemoglobin was observed respectively, whereas only 1.74 μM of oxyhemoglobin was released when untreated erythrocytes were exposed to free radicals (Fig. 64). Almost no release of oxyhemoglobin was observed in erythrocytes, which were neither treated with lectin nor exposed to oxidative damage (control), or lectin treated cells but not exposed to oxyradical shock.

Effect of lectin on hypochlorous acid induced hemolysis

The oxidizing action of HOCl was monitored in the presence of BBL in terms of percent erythrocytes hemolysis. As evident in Fig. 65, the extent of cell hemolysis

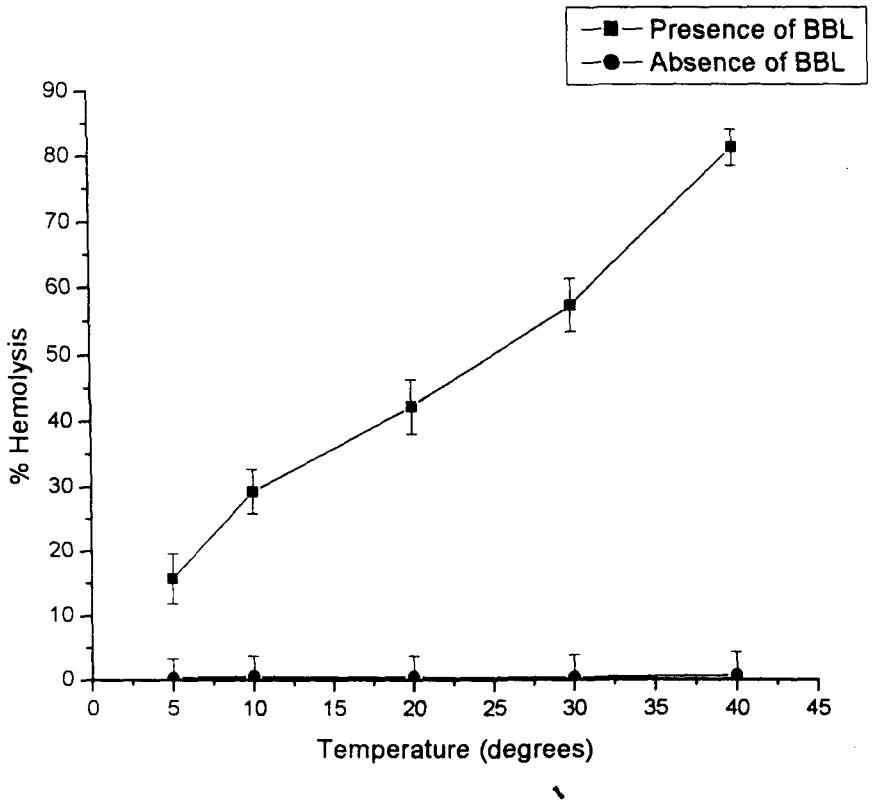


Figure 60. Effect of temperature on BBL induced hemolysis of trypsinized rabbit erythrocytes.

The erythrocytes treated with lectin at a concentration of 100μg/ml in 75 mM PBS pH 7.4, were incubated at different temperatures for 4 hours. Values shown are the mean ± S.D obtained from three observations.

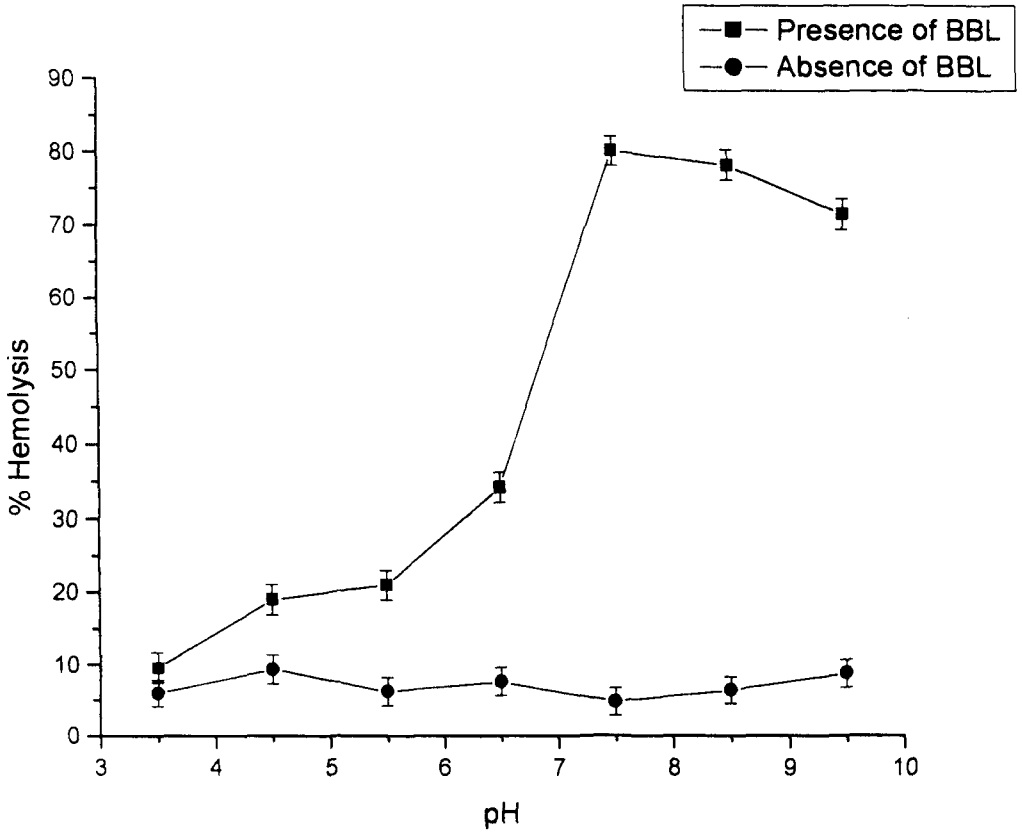


Figure 61. Effect of pH on BBL induced hemolysis of trypsinized rabbit erythrocytes.

Trypsinized erythrocytes treated with lectin (100μg/ml) in 75 mM PBS pH 7.4, were incubated in various buffers (0.1 M sodium acetate/sodium phosphate/Tris-HCl/ glycine NaOH) of varying pH values (3.5-9.5) at 37°C for 4 hours. Values shown are the mean ± S.D obtained from three observations.

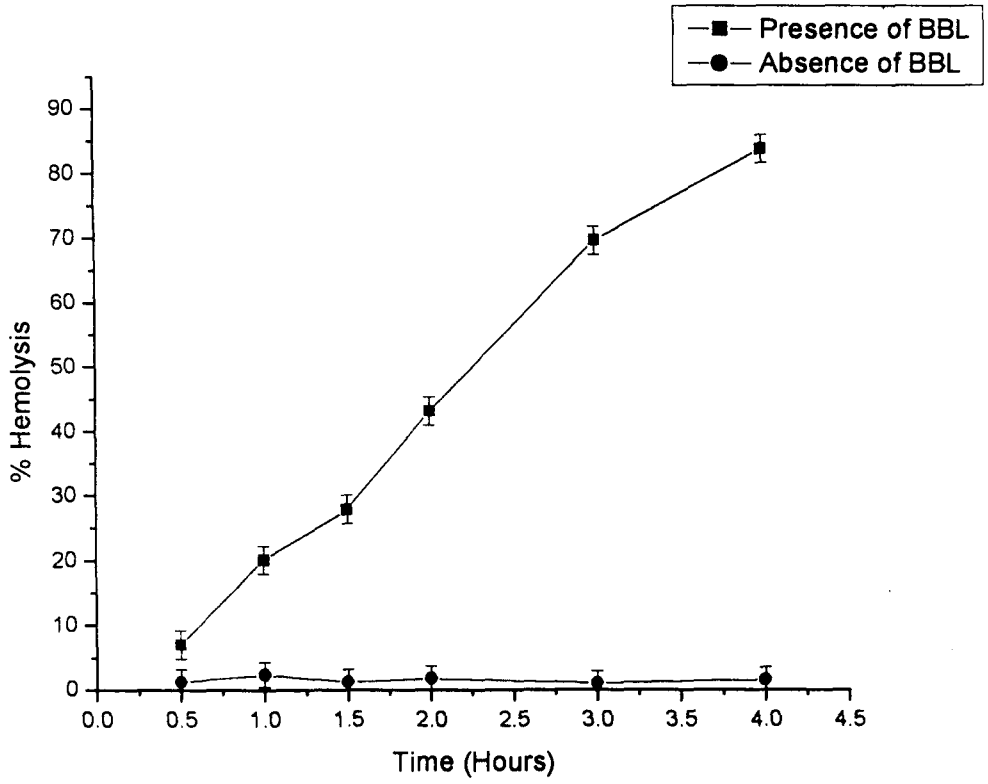


Figure 62. Effect of incubation time on buffalo lectin induced hemolysis of trypsinized rabbit erythrocytes.

The erythrocytes treated with lectin (100μg/ml) in 75 mM PBS pH 7.4, were incubated for different time intervals (0.5-4 hours) at 37°C. Values shown are the mean ± S.D obtained from three observations.

was directly proportional to HOCl concentrations. Prior agglutination of erythrocytes with BBL resulted into significant ($P<0.001$) enhancement of hemolysis as compared to untreated cells in the presence of varying concentration of HOCl. At 350 μM NaOCl concentration the BBL agglutinated erythrocytes showed 47.46 % hemolysis which was 42.99% more than the non-agglutinated cells (33.19 % hemolysis).

Differential hemolytic action of lectin towards erythrocytes of cancerous patients

Lectin-mediated agglutination of erythrocytes of cancer patients showed a differential pattern of hemolytic activity when compared to healthy control. Hemolysis was considerably increased (50.58%) in the presence of lectin in RBCs from normal healthy control (2.11%) (Fig.66). The extent of hemolysis in BBL treated erythrocytes from pre-operated (breast cancer) samples did not show an appreciable increase (30.33 %), whereas, there was a significant ($P < 0.001$) increase of 41.5 % in the % hemolysis in post-operated breast cancer RBC's.

In case of prostate cancer patients, agglutination with BBL displayed an increase in % hemolysis in both pre-operated (75.4%) and post-operated (54.90%) erythrocytes with respect to unagglutinated pre-operated (55.96 %) and post-operated (13.05 %) RBC's (Fig. 67).

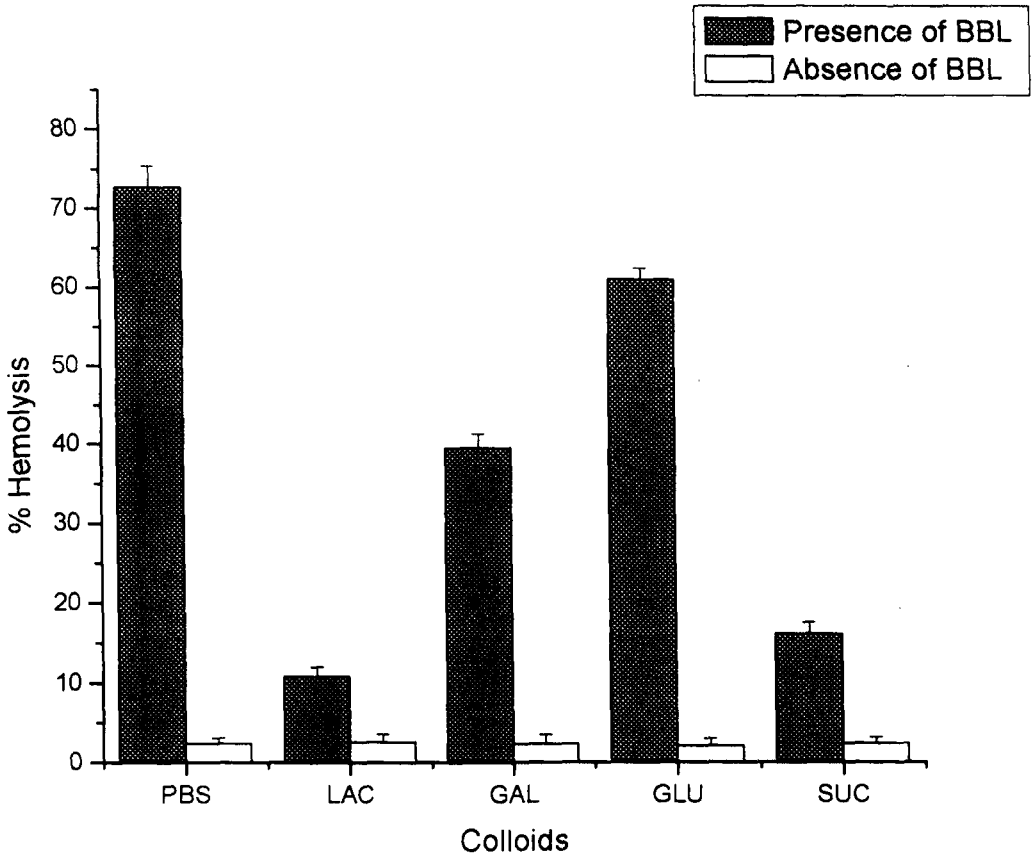


Figure 63. Osmotic protection against BBL induced lysis by various carbohydrates.

The hemolysis of erythrocytes was measured in the presence of 30 mM of various sugars in 75 mM PBS pH 7.4. Phosphate buffered saline (PBS), Lactose (Lac), Galactose (Gal), Glucose (Glu), Sucrose (Suc). The measurements were performed in triplicates with 10% rabbit erythrocytes and 100μg/ml buffalo brain lectin in PBS pH 7.4. Values shown are the mean ± S.D obtained from three observations.

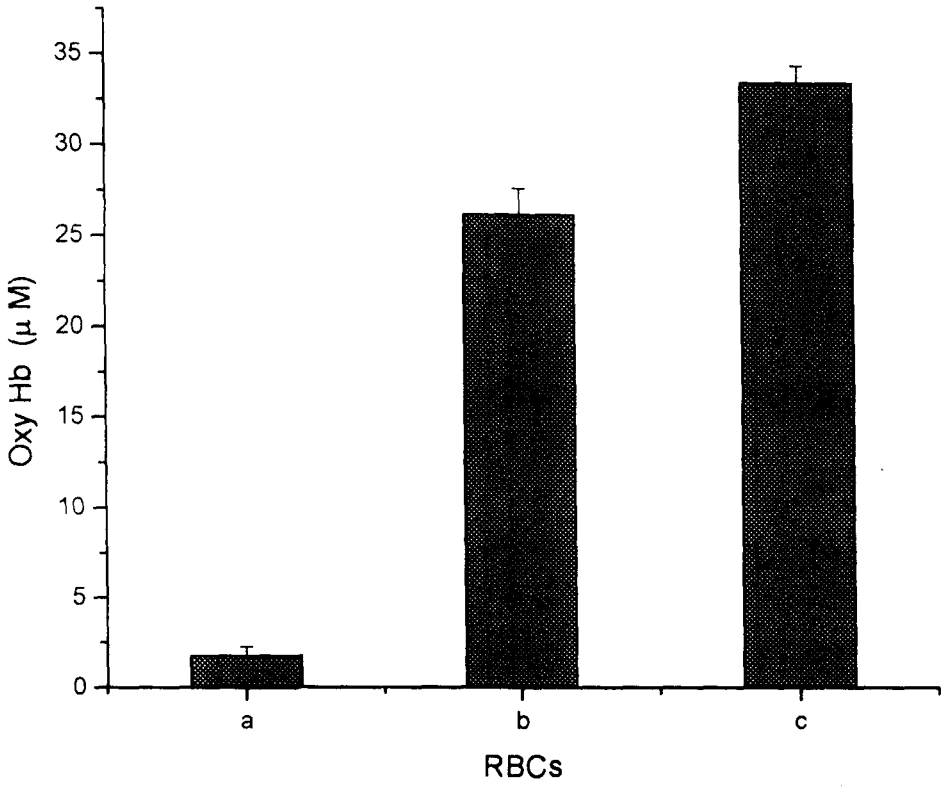


Figure 64. Measurement of OxyHb concentration under various conditions in the hemolysates of a; erythrocytes exposed to superoxide radicals, b; erythrocytes exposed to superoxide radicals in the presence of BBL after 1 hour, c; erythrocytes exposed to superoxide radicals in the presence of BBL after 4 hours. Values shown are the mean ±S.D obtained from three observations. No oxyHb was released in the erythrocytes (absence /presence of BBL) not exposed to superoxide radicals.

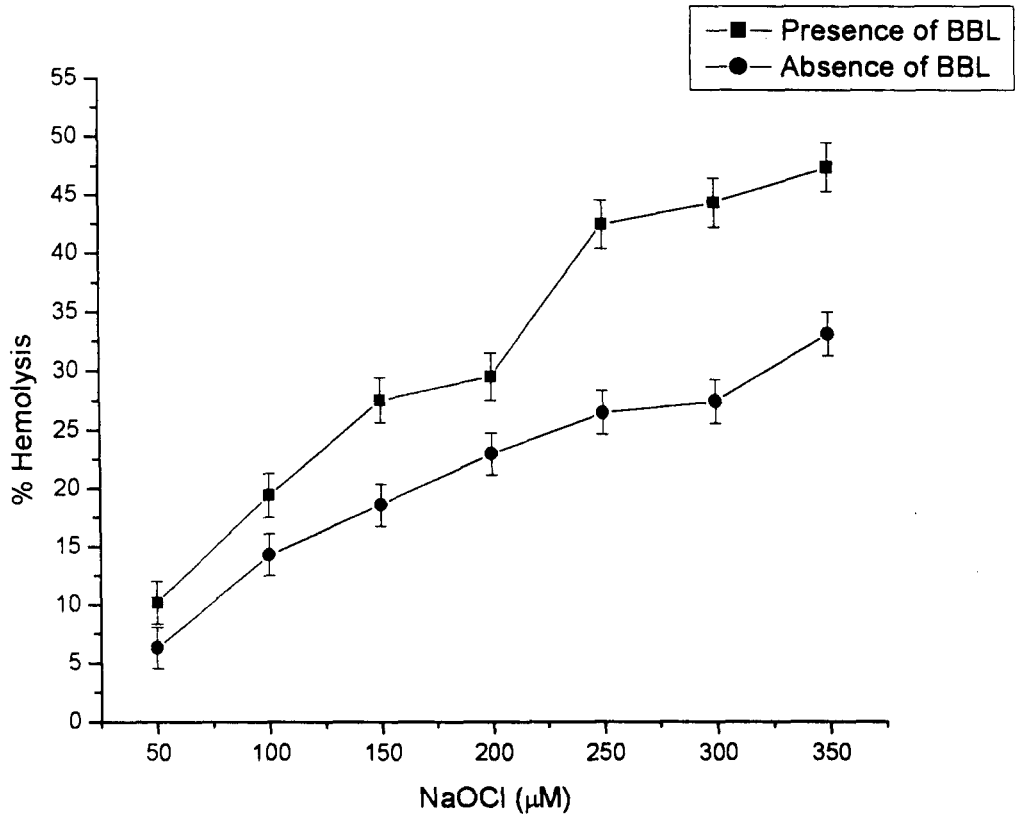


Figure 65. Effect of BBL on hypochlorous acid induced hemolysis.

The erythrocytes treated with lectin (50μg/ml) were incubated at different concentration of NaOCl in 75 mM PBS pH 7.4. Values shown are the mean ± S.D obtained from three observations.

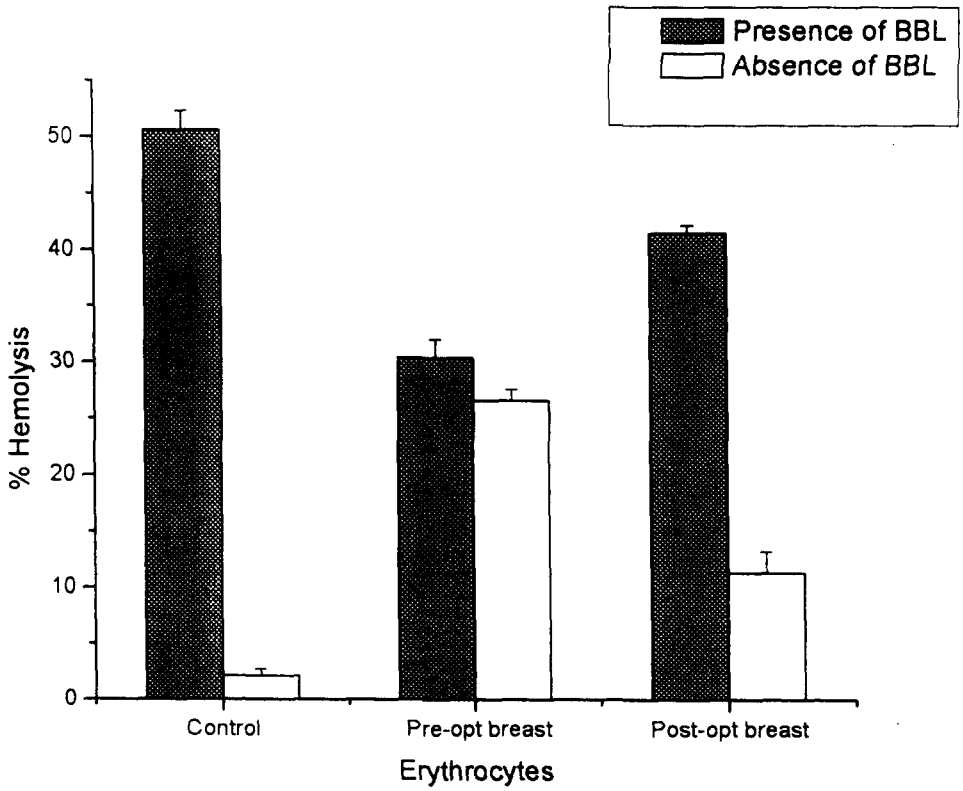


Figure 66. Hemolysis of human erythrocytes of normal, pre-operated and post-operated breast cancer patients in the presence and absence of BBL (50 µg/ml).

The degree of hemolysis was calculated by comparing with identical volume of erythrocytes mixed with distilled water which represented 100% lysis. Results are reported as mean of six independent readings ± S.D.

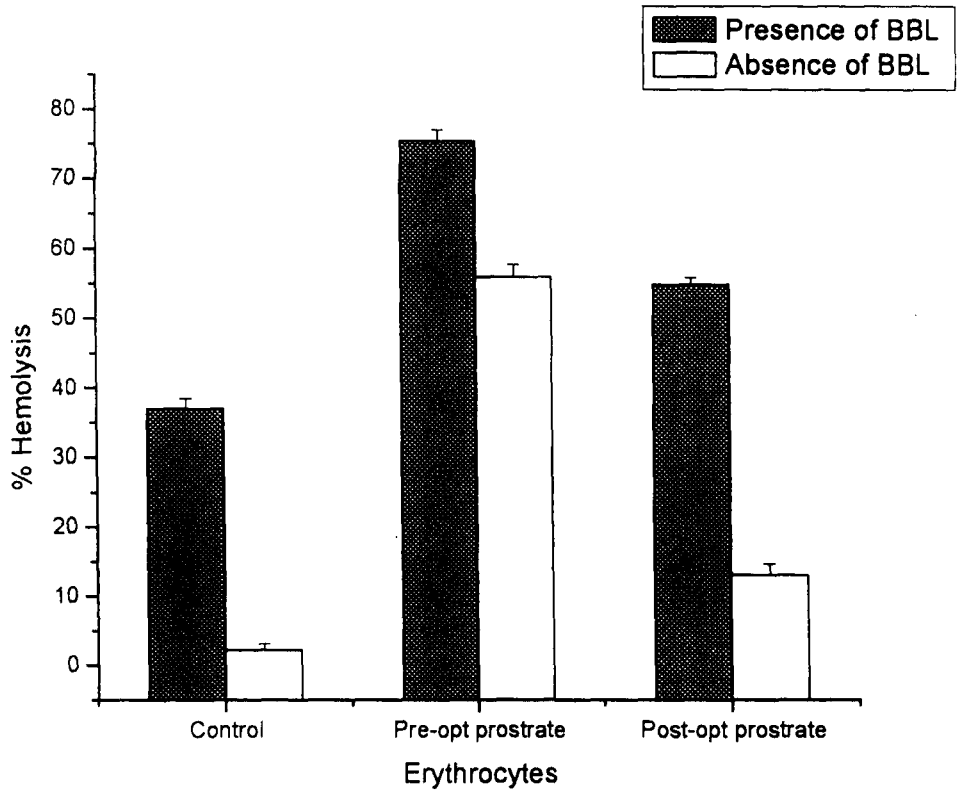


Figure 67. Hemolysis of human erythrocytes of normal, pre-operated and post-operated prostate cancer patients in the presence and absence of BBL (50 µg/ml).

The degree of hemolysis was calculated by comparing with identical volume of erythrocytes mixed with distilled water which represented 100% lysis. Results are reported as mean of six independent readings ± S.D.

Discussion

DISCUSSION

Lectins are characterized by their non-immunoglobulin origin and specific carbohydrate binding property that does not display any enzymatic activity towards the recognized sugar moiety. Soluble β-galactoside binding lectins have been isolated, studied and characterized from variety of sources ranging from *C. elegans* (Hirabayashi et al., 2002) to various mammalian species, expressed in an array of different tissues like muscles (Child and Feizi, 1979), heart (Waard et al.,1976) lungs (Waard et al., 1976) skin (Bols et al., 1986) and brain (Caron et al., 1990). Several workers have investigated and characterized the presence of β-galactoside binding lectins from neural tissues of different mammals (Kuchler et al., 1989, Caron et al., 1990, Zanetta et al., 1992, Jaison and Appukuttan, 1999; Ola et al., 2001; Shahwan et al., 2004) where they reside in a subpopulation of dorsal root ganglion, primary sensory neurons, motor neurons, astrocytes, perivascular cells and microvessels (Joubert et al., 1989; Hynes et al., 1990; Horie et al., 2004; Stillman et al., 2005; Stillman et al., 2006).

The present work is a comprehensive study which describes the purification and characterization of soluble lectin having affinity for β-galactoside residues from the nervous tissue of water buffalo (*Bubalus Bubalis*). The brain tissue extract rapidly agglutinated trypsinized rabbit erythrocytes, thus displaying the presence of lectins. The present scheme of purification involves a combination of ammonium sulphate precipitation and gel filtration chromatography on Sephadex $G_{50\text{-}80}$ column. The ammonium sulphate fractionation resulted in partial purification and enrichment of specific activity of brain lectin. Moreover, gel permeation on sephadex $G_{50\text{-}80}$ was quite effective in complete purification of protein as a single peak with high fold purification, thus making the entire procedure rapid, simple which gave a comparable yield of 0.04 % with others workers (Caron et al., 1987; Bladier et al., 1991; Ola et al., 2001).

The purified lectin migrated as a single band in non-denaturing PAGE as well as SDS-PAGE under reducing conditions suggesting the homogeneity of the preparation. The subunit molecular weight of BBL corresponded to 14.5 kDa both in reducing as well as non reducing conditions, similar to the lectin isolated from bovine, rat and human nervous tissue (Caron et al., 1987, Bladier et al., 1991). Moreover, in size exclusion chromatography the native lectin behaved as a globular protein with

molecular weight of 28.5 kDa suggesting that it is presumably a homodimeric protein with two identical subunits held together by non-covalent interactions. These results were consistent with the values obtained for the lectin from nervous tissue of other mammalian species (Cerra et al., 1985; Caron et al., 1987; Bladier et al., 1991).

The dimeric structure was further confirmed by the Scatchard analysis, which indicated the presence of two carbohydrate binding sites per native molecule of lectin. This result was quite comparable to that obtained for bovine spleen galectin (Ahmed et al., 1996). Association constant (K_{ass}) for the binding of lactose to BBL was approximately 6.66×10^{3} M^{-1} which is slightly lower than that of electrolectin (Levi and Teichberg, 1981). The dimeric form of β-galactoside binding lectins provides them the potential to bind to glycoconjugates. It is reported that galectin-1 also causes biphasic modulation of cell growth (Stillman et al., 2005). The positive and negative effects of galectin-1 on cell growth might be influenced by both dose and the relative distribution of monomeric versus dimeric form (Camby et al., 2006; Stillman et al., 2006).

Most β-galactoside binding lectins display affinity towards lactose and N-acetyllactosamine with subtle differences in their carbohydrate specificities (Oda et al., 1993; Ahmed and Vasta, 1994; Leffler et al., 2004; Vasta et al., 2004; Rabinovich et al., 2007). Therefore, a number of simple sugars and complex saccharides were screened for sugar specificity of BBL by hemagglutination inhibition assay. BBL displayed its carbohydrate specificity profile similar to the known brain lectins from bovine, rat, goat, sheep and human (Leffler and Barondes, 1986; Caron et al., 1987, Bladier et al., 1991, Ola et al., 2001, Shahwan et al., 2004) with greater affinity for disaccharides, in particular for lactose. The minimum inhibitory concentration required for other saccharides was also different, suggesting that the BBL has its own unique and fine specificity (Sparrow et al., 1987; Solis et al., 1996, Kasai and Hirabayashi, 1996; Rabinovich et al., 2007). Lectin obtained from buffalo brain was maximally inhibited by lactose, suggesting its specificity for carbohydrate moieties in β-glycosidic linkages. Thus, hemagglutination inhibition assay clearly demonstrated that BBL is specific for saccharides bearing non-reducing terminal galactose linked in a β-configuration. This result was further confirmed by weak binding of BBL to methyl-α-D-galactopyranoside and p-nitrophenyl- α-D-galactopyranoside as compared to methyl-β-D-galactopyranoside and p-nitrophenyl-β-D-galactopyranoside. In addition, nitrophenylated galactose was a more effective

inhibitor of BBL activity than galactose or methyl-β-D-galactopyranoside, suggesting the involvement of hydrophobic interaction in the saccharide binding (Ali and Salahuddin, 1989; Ola et al., 2001). Moreover, D-galactose and D-galactosamine inhibited the hemagglutination reaction, indicating the significance of free hydroxyls or a free amino group at C-2 in monosaccharides to cause inhibition of BBL binding to erythrocytes (Ola et al., 2001; Shahwan et al., 2004). The hemagglutinating activity was unaffected by the presence of sugars like mannose, sucrose, melibiose, fucose and fructose. However, the inability of glucose or glucosamine to cause inhibition sheds light on the importance of C-4 configuration. The extended like geometry of carbohydrate recognition domain of BBL is suggested due to its high affinity for lactose in comparison to galactose which gets only partially occupied upon galactose binding (Loris, 2002; Rabinovich et al., 2004). This is quite consistent with the X-ray crystallographic studies using small disaccharides which predicted the 4-OH and 6-OH groups of galactose and 3-OH group of glucose comprising lactose structure are critically important for high affinity binding (Lee et al., 1990; Liao et al., 1994; Loris et al., 2002).

Gal-1 is a non-covalent homodimeric protein composed of 14.5 kDa subunits consisting of two identical conserved carbohydrate recognition domains. Thus, BBL isolated in the present study is a β-galactoside binding protein resembling the Gal-1 family in functional and structural respects, but inclusion of this lectin in the galectin-1 family still awaits the sequence analysis for the presence of sequence similarity in CRD domains.

BBL readily agglutinates all types of native human erythrocytes apart from agglutination of trypsin treated human blood cells. It agglutinated equally both blood group A and O erythrocytes, probably mediated by the polylactosaminoglycans found on the surface of human erythrocytes (Fukuda, 1985; Ola et al., 2001).

It is a well established fact that almost all the known β-galactoside binding lectins require reducing agents for the hemagglutination activity and stability (Cerra et al., 1985; Southan et al., 1987; Horie et al., 2004). BBL also strictly requires reducing agents such as β-ME to show activity for longer period of time. The chemical analysis of BBL also revealed the presence of 2.8 thiol groups per mole of BBL. Thus in order to investigate whether free thiol groups were necessary to maintain lectins in their active form, the native BBL was alkylated by iodoacetate and iodoacetamide. This led to a wide range in rates at which thiol groups were alkylated and resulted in

inactivation of hemagglutination activity, suggesting the need for free thiol groups for the maintenance of BBL in the active form. Thus, the loss in the lectin activity could be due to a possible conformational change that occurred during modification of cysteine residues (Clerch et al., 1988), whereas the slow rate of inactivation reflects that the cysteine residues could be partially buried inside the protein and possibly not involved directly in carbohydrate binding but present at a relatively distant site (Whitney et al., 1986; Shahwan et al., 2004). It is also surprising to observe the ready inactivation of buffalo lectin by iodoacetamide contrary to the absence of inactivation or display of higher activity in the presence of iodocetamide by other lectins (Whitney et al., 1986; Clerch et al., 1988; Shahwan et al., 2004).

In addition, BBL was irreversibly inactivated by exposure to high temperature during a relatively short period of time in contrast to bovine galectin which was found to be more resistant to thermal inactivation as it retained 6% of total activity even at 100°C for 30 min (Ahmed et al., 1996). The BBL was quite stable between pH 6.5-9.5 and overlapped with that of natural physiological environment as reported earlier for human spleen galectin (Ahmed et al., 1990; Ahmed et al., 1996).

The functional and structural integrity of BBL was assayed in the presence of various detergents in order to highlight the susceptibility of brain proteins towards different denaturing media. SDS is a well known ionic detergent which denatures proteins by ordering protein water molecule around them and also by intercalating into hydrophobic protein clefts with its long hydrocarbon chain (Lanio et al., 2003). Thus, the present study too demonstrated the denaturing effect of SDS on BBL even at a very small concentration. Tween-20 and Triton X-100 being neutral detergents were less inhibitory on BBL as they do not bind with lectins in detectable amount and were not very efficient in breaking non-covalent bonds which are responsible for the quaternary structure of multimeric proteins (Helenius and Simons, 1972). However, detrimental action of detergents was largely prevented by the presence of lactose which possibly excludes detergents from BBL sites susceptible to denaturation and proteolysis (Doyle et al., 1973; Jollifes et al., 1980; Denson and Doyle, 1998).

Interestingly, our findings also showed that mammalian β-galactoside binding lectins are potent inducers of mammalian brain cell aggregation. It is important to note that lectin carbohydrate binding need not be the only or even the main determinant of specificity in brain cell interaction. A requirement for dual recognition involving other

sets of complementary molecules is consistent with the lectin recognition hypothesis (Caron et al., 1987). This result may also imply the involvement of the brain galectins in neurotransmission process and neuronal cell differentiation (Stillman et al., 2005). The spectroscopic analysis of BBL showed that lectin fluoresces at 335 nm and has its UV maximum at 282 nm, confirming the presence of aromatic residues particularly tryptophan moiety in a hydrophobic environment. The Far-UV CD spectra of native BBL corresponds to that of a protein with a high content of β-sheet with a minimum around 217 nm, typical to other β-galactoside binding lectins as confirmed by X ray crystallography (Liao et al., 1994; Horie and Kadoya, 2004; Houzelstein et al., 2004). An FTIR spectrum also correlates with CD analysis, consistent with the presence of large extent of β-sheet structure as suggested by maximum absorbance peak at 1635 cm^{-1}.

Nearly all known β-galactoside binding lectins with few exceptions require reducing agents for their activity (Southan et al., 1987; Ola et al., 2001; Kadoya et al., 2005; Rabinovich et al., 2007). Buffalo brain lectin also shows hemagglutination activity only in the presence of reducing agents such as β-mercaptoethanol. Purified lectin showed no detectable loss of activity when kept for up to 3 months in PBS containing 0.3 M lactose at 4°C, thus, suggesting that lactose maintains the purified protein in the active form even in the absence of a reducing agent, possibly by preventing oxidative inactivation due to formation of intra-sulphide linkages (Cho and Cummings, 1995). Since these lectins remain in active conformation in the presence of a reducing agent, it led us to believe that they contain an oxidizable residue whose integrity is quite crucial for its activity. Our results clearly demonstrate that the exposure of lectin to an oxidant causes a decrease in its activity which can be due to the oxidation of either cysteine or tryptophan residue. Oxidation of cysteine residue can be possibly ruled out as UV absorption maxima in the presence of an oxidant showed a sharp decline from 282 nm to 250 nm, typical to that of an oxidized Trp residue (Patchornik et al., 1960). Oxidation of Trp residue was further confirmed by quenching of the intrinsic fluorescence of the protein in the presence of 5 mM H_2O_2 with a slight blue shift. In addition, the decrease in the fluorescence of BBL was quite concomitant with the loss of activity at different time intervals. Apparently, H_2O_2 did induce subtle changes in lectin conformation in such a way that fluorophor is transferred to less polar environment and also led to the oxidation of tryptophan residue to form an oxindole. Moreover, the finding that lactose increased the fluorescence of lectin and prevented

the deleterious effect of H_2O_2 indicated that the emitting fluorophore is located within the lactose-binding site (Levi and Teichberg, 1981). Since, the tryptophan fluorescence is quenched by H_2O_2, the loss of lectin activity can be accounted for by the oxidation of the tryptophan residue present in the lactose-binding site, whereas, in the presence of lactose, oxidation of the tryphtophan residue and formation of oxindole is prevented. Thus, due to high susceptibility of brain lectins to oxidation they are often advised to be stored in lactose solution. These findings suggests the importance of reducing environment which is probably needed to reduce molecular oxygen normally present in solution and prevent it from oxidizing tryptophans (Levi and Teichberg, 1981). Although, there is no direct evidence that tryptophan is crucial for hemagglutination activity in case of buffalo lectin, but it may play some possible role in maintenance of the carbohydrate binding site of the lectin molecule as several workers based on X-ray studies on bovine spleen galectin have shown that Trp-68, a highly conserved amino acid in β-galactoside binding lectins is found to make stacking interaction with galactose moiety (Abbot and Feizi, 1991; Hirabayashi and Kasai, 1991; Rini and Lobsanov, 1999; Vasta et al., 2004).

The effect of oxidant was also monitored by circular diochroism and FTIR analysis. The native secondary structure of BBL underwent a major transition from β-pleated form into a more open and enriched α-helix conformation (as evident by a shift in CD spectra and FTIR spectra), suggesting the reason for the loss of activity upon oxidation (Pande et al., 2003; Kadoya et al., 2005). An increase in α-helix content has also been reported for galectin in the presence of H_2O_2 (Pande et al., 2003; Shahwan et al., 2004), which owing to disulfide bond formation possibly locks the protein into a new inactive conformation that cannot form the usual secondary structure, thereby, losing its potential to bind saccharides (Clerch et al., 1988; Pande et al., 2003; Horie and Kadoya, 2004). This suggests that the regular secondary structure is vital for maintaining the active conformation of lectin.

Near-UV CD spectra of BBL captured under the same conditions showed a significant change in the tri-dimensional structure of the protein, indicating a non-native structure and Trp spatial disposition in BBL as discussed previously by fluorescence studies. In particular, the data obtained shows a total loss of tertiary structure which is compatible with a more open conformation.

Presence of lactose does not bring any significant change in secondary and tertiary topology of BBL as revealed by CD and FTIR analysis. Slight changes in spectras (CD

and FTIR) may be contributed by the alterations in the tryptophan residues. However, lactose does play a preventive role against deleterious effect of H_2O_2. Far-UV CD and FTIR analysis of BBL pre-incubated with lactose in oxidizing conditions showed a slight protection against the secondary conformational change. However, near-UV analysis displayed a significant difference between the lactose treated BBL and untreated BBL in the presence of H_2O_2. These striking augmentations correlate with the earlier fluorescence studies where lactose prevented the exposure of tryptophan residue to oxidizing media. Probably, a stable conformational state is achieved in the presence of specific sugars, which involves the masking of tryptophan residue or the provocation of some conformational changes in the lectin resulting in the internalization of tryptophan residue (Khan et al., 2001).

The exact biological action of 14-kDa lectin in brain is not yet known. Recent evidences indicate that the brain lectins are involved in the potentiation of neuropathic pain in the dorsal horn of rat olfactory system (Puche and Key, 1995; Kadoya et al., 2005; Stillman et al., 2006). However, it has been demonstrated that oxidized β-galactoside binding lectin enhances axonal regeneration in the peripheral nervous system but does not posses hemagglutinating properties (Inakaki et al., 2000; Horie and Kadoya, 2004; Stillman et al., 2005; Stillman et al., 2006). Our studies also demonstrate that oxidized form is totally inactive thus, proving that oxidized functional lectin in the nervous tissue may just acts as an autocrine or paracrine factor, functioning more like a cytokine or chemokine than as lectin. Thus, this information opens new insights into the functions of galectins in mammalian nervous system.

The denaturation of BBL by various chaotropic denaturants (Guanidine HCl (GdnHCl), urea and thiourea) indicated that its globular nature is possibly stabilized mainly by hydrogen binding and hydrophobic interactions (Nelson and Cox, 2001). The conformational changes induced by different denaturants were also monitored by fluorescence spectroscopy. The fluorescence emission spectrum of BBL in the presence of increasing concentrations of GdnHCl showed a decrease in the fluorescence intensity indicating that the tryptophan residue in native protein is located near an intra-molecular quenching group and the denaturation of the protein increases this interaction. These changes were also accompanied by a red shift from 340 to 357 nm at different concentrations of GdnHCl. This shift results from the extent of exposure of the Trp residue to bulk solvent. Higher denaturant concentrations, up to 6 M GdnHCl had no effect on the position of the maximum but

a slight increase in fluorescence intensity was observed. In contrast, the fluorescence emission spectrum of BBL in the presence of increasing concentrations of urea and thiourea showed an increase in the fluorescence intensity suggesting the exposure of tryptophan residue to a more polar environment. In particular, the increase observed at various concentrations of denaturants could reflect an unfolding of the protein, thereby, preventing the quenching of the fluorescence by the polar solvent (Friedfelder, 1935). Increase in fluorescence intensity was also accompanied by a red shift at different concentrations of urea and thiourea. Thus, in all, BBL undergoes a denaturants-induced alteration in its conformation that involves a modification of the environment of the Trp residues (Freidfelder, 1935; Iglesias et al., 2003).

Although galectins are often reported to be present on cell surfaces or in extracellular matrix, they lack recognizable secretion signal sequences and do not pass through the standard endoplasmic reticulum/Golgi pathway (Hughes, 1999), with the possible exceptions of a sponge galectin (Miarons and Fresno, 2000). As a result most of the isolated galectins have characteristics typical of cytoplasmic proteins, such as an acetylated N-terminus, free sulfhydryls and lack of glycosylation (Hughes, 1999). Thus, β-galactoside binding lectins are usually non-glycosylated proteins but interestingly, carbohydrate analysis of BBL revealed the presence of 3.3 % sugar moiety of the total mass of the lectin. Till date, all the mammalian brain lectin isolated are found to be devoid of any carbohydrate moiety (Bladier et al., 1989; Caron et al., 1990; Ola et al., 2001; Shahwan et al., 2004). Therefore, it was interesting to study the role of glycosylation on the structure and functions of lectin by a cooperative analysis of its activity and structural stability in both glycosylated and deglycosylated forms over a wide range of temperature, pH, and in the presence of various denaturing agents.

The addition of large glycan structures to the protein backbone can dramatically alter the structure, and consequently the function of the polypeptide architecture to which they are attached (Sinha and Surolia, 2007). Linked glycans can affect protein structure in two capacities; firstly addition of the carbohydrate to the partially folded nascent polypeptide can have an impact on, or facilitate the protein-folding process. Secondly, the carbohydrate can stabilize the mature protein (Wang et al., 1996; Sinha and Surolia, 2007). Therefore, in order to study the role of carbohydrate residue on soluble lectin, we removed the sugar residues using periodate oxidation method. This method completely removed the sugar moieties which were confirmed by the Dubois

analysis and the difference in the mobility of the native and modified protein on SDS-PAGE. Consistent with other workers (Wang et al., 1996; De Koster and Robertson, 1997), thermal stability of native lectin proved to be higher than its deglycosylated form over a wide range of temperature. The thermal denaturation of both forms of BBL at 60°C for various time intervals showed greater retention of hemagglutination activity for glycosylated native lectin. Thus, presence of carbohydrate residues to the protein backbone clearly indicates a protective role in thermal melting of proteins (Wang et al., 1996; Arnold and Hoffman, 1997).

There was not any change in the pH optima of glycosylated and deglycosylated forms when their activity was tested as a function of pH, however, a decreased activity profile was observed for deglycosylated lectin. Thus, the presence of carbohydrate moiety protected the protein from extreme conditions of pH (Wang et al., 1996; Rasheedi et al., 2003).

Deglycosylated lectin showed lower hemagglutinating activity as compared to the native glycosylated BBL when treated with different concentrations of various denaturants and detergents. Earlier reports have already shown the role of carbohydrate residue in imparting resistance to denaturants mediated inactivation (Rasheedi et al., 2003; Fatima and Hussain, 2007).

The deglycosylated form exhibited higher fluorescence intensity as removal of carbohydrate residues resulted in a greater exposure of the flurophore, concomitant with earlier reported studies relating to fluorescent behavior of glycosylated and nonglycosylated proteins (Rasheedi et al., 2003; Jafari-Aghdam et al., 2005; Fatima and Hussain, 2007). The fluorescence studies with different denaturants revealed the unfolding of the native and deglycosylated form. The fluorescence emission spectrum of BBL in the presence of increasing concentrations of GdnHCl showed a decrease in the fluorescence intensity indicating that the tryptophan residue in native protein is located near an intramolecular quenching group and the denaturation of the protein increases this interaction. The decrease was observed till 6 M concentration above which there was a slight increase in the fluorescence intensity. These changes were also accompanied by a red shift from 335 nm to 357 nm which resulted from the extent of exposure of the Trp residue to bulk solvent. At each concentration of GdnHCl, the deglycosylated lectin exhibited higher fluorescence intensity and a greater red shift as compared to the native BBL. Thus, carbohydrate moieties due to steric hindrances play a significant role in shielding the trytophan residues of native

lectin from more polar microenvironment (Fatima and Hussain, 2007).

While, in the presence of urea and thiourea there was an increase in the fluorescence intensity with a pronounced red shift for both forms of lectin, with the deglycosylated lectin exhibiting higher intensity as compared to its native glycosylated form. This again highlights the importance of the glycan residues attached to the native protein which stabilizes its structure and protects the unfolding of the protein. Glycosylation has been reported to increase the stability of a number of molecules against different denaturating agents (Khan et al., 2003; Rasheedi et al., 2003; Sinha and Surolia, 2007) and increased resistance against protease degradation (Van Berkel et al., 1995). Thus, there was an overall change in the structure of both the forms and a reorientation of the tryptophan residues in the presence of different denaturants, which was more pronounced in deglycosylated form. Based on these studies, it can be predicted that glycosylation influences the conformational dynamics of nascent polypeptides and confers their biological activity.

To the best of our information, as no mammalian glycosylated lectin has been reported so far (Bladier et al., 1989; Caron et al., 1990; Ola et al., 2001; Shahwan et al., 2004), the study of this glycosylated lectin may prove to shed some light on the significance of sugar residues on soluble lectin and ultimately enhance knowledge of the already growing repertoire of mammalian lectins.

In view of the possible role of brain lectins, it was of interest to see whether the isolated lectin is phylogenetically/structurally related to other brain lectin or is a distinct protein sharing only a similar saccharide-binding specificity. Lectin obtained from buffalo brain was highly immunogenic in rabbits as it readily gave a single line of identity when tested by double immunodiffusion indicating the homogeneity of protein preparation. Direct binding ELISA was performed to characterize the immune response in rabbits using pure buffalo brain lectin as an antigen. The antiserum showed a high titre >128000 again suggesting the high immunogenic nature of BBL. Preimmunized serum served as negative control and did not show any appreciable binding to buffalo brain lectin.

Antigenic crossreactivity between buffalo, sheep and goat lectins by immunodiffusion studies show that anti BBL antibodies recognized both goat and sheep brain lectins, but comparatively faint precipitin lines were observed with respect to buffalo brain lectin indicating that antibody was not highly specific to sheep and goat lectin as compared to buffalo lectin. The specificity of the induced

antibodies and sharing of common antigenic determinants between lectins was further ascertained by competition ELISA using purified sheep, goat and buffalo brain lectins as competitors. The induced antibodies showed a high degree of specificity for buffalo brain lectin (immunogen), however, the extent of binding was lower for sheep and goat lectins. The buffalo brain lectin inhibited the anti buffalo antibody binding to solid phase bound antigen by 87%, while the inhibition of goat and sheep lectin was 53% and 62%, respectively, depicting that buffalo brain lectin has a slightly low degree of antigenic similarity or conformational homogeneity with sheep and goat brain lectins. The antibodies were quite specific for the immunogen (BBL), since, only 5 μg/ml of BBL was required for achieving 50% inhibition. A low level of cross reactivity of anti-buffalo lectin antibody with goat and sheep was again confirmed as concentration required to achieve 50% inhibition corresponded to 18 μg/ml and 9.5 μg/ml for goat and sheep lectins, respectively and the relative affinities for goat and sheep lectins were found to be 60.9 % and 71.26 %, respectively. Thus, the overall results indicate that lectins from different species share only few common antigenic determinants or epitopes. Antigenic reactivity between the sheep and goat brain lectins corroborates with the earlier findings and is consistent with the results for species which are phylogenetically related (Ola et al., 2001; Shahwan et al., 2004). These observations on the cross reactivity between brain lectins of different species suggest a taxonomic variation in structure and function of these lectins analogous to that observed with cytochrome C (Dickerson and Geis, 1969; Beyer et al., 1979).

To check the possible structural similarity of buffalo brain with similar lectins present in other organs of buffalo, the antiserum was tested against various tissue homogenates like heart, liver and lung by dot blot analysis. It was found that antibodies reacted strongly with all of them. These results indicated the antigenic similarity between buffalo brain lectins and similar lectins from other organs of the same specie (Beyer et al., 1979).

The exact biological function of brain lectin is still obscure, yet recent reports suggest that they have a specific role in nerve degeneration (Kadoya et al., 2005; Camby et al., 2006) and development of brain carcinoma (Stillman et al., 2005; Stillman et al., 2006). However, the localization of lectins in different tissues has also brought up the suggestion that the endogenous lectin may play different roles in different organs (Beyer et al., 1979). It seems more likely that they play a common

role in different cell types as recognition molecules for cellular moieties containing carbohydrate structure with terminal β-galactosyl residues (Beyer et al., 1979). Our results showing antigenic cross reactivity between lectins from different tissues and different species are certainly in line with the idea that all the β-galactoside binding lectins in animal tissues have a common fundamental biological role that has been preserved through evolution (Levi and Teichberg, 1982; Ola et al., 2001).

Proteins with hemolytic or in general with cytolytic activites have been of much interest to membrane biologists, since, the study of the mode of action of such cytolytic agents has contributed considerably to the understanding of complexities of biomembrane organization and function. Interestingly, β-galactoside binding lectin from buffalo brain also exhibited hemolytic activity towards rabbit and human erythrocytes. Lytic actions of some proteins have been ascribed to enzymatic activity (Hittelet et al., 2002), perturbation of the activities of membrane associated enzymes (Lowe and Marth, 2003), or pore formation in the membranes (Yu et al., 2002). Thus, we examined the interaction of BBL with the erythrocyte membrane in order to elucidate the mechanism of hemolysis by BBL using rabbit erythrocytes as a model system. The study showed that BBL agglutinated RBCs were significantly hemolysed in comparison to unagglutinated RBCs, suggesting that lectin by virtue of its glycan binding property creates pores or leaks in the cellular membranes and thereby disrupts its function as a selective cell barrier. Thus, due to an imbalanced colloid-osmotic pressure of intracellular solutes (Devticke et al., 1986) uptake of water and salts rises, causing an increase in hemolysis in lectin agglutinated cells. Similar results have also been obtained for plant and animal lectins where they interact with membrane glycoconjugates and penetrate the lipid bilayer, thereby disturbing its integrity and fluidity (Hajela et al., 1997; Pande et al., 1998; Gupta et al., 2006). Lectins, upon contact with cell surface glycans like laminin, fibronectin and lysosyme associated proteins, oligomerize into dynamic complexes that have high on or off rates of interaction with the lipid bilayer, which ultimately results in substantial reorganization of membrane components (Surolia et al., 1997). Contact point of lipid bilayer and membrane integrated proteins is tightly intact and any displacement of membrane proteins due to the lectin binding may disturb the sealed interaction, thus, establishing an aqueous pore (Israelachivilli, 1977). Aqueous trans-membrane channels with hydrophobic protein domain coated walls may also form with a cluster of aggregated and displaced proteins (Pande et al., 1998; Gupta et al., 2006).

A rise in temperature increases the rate of electrolyte diffusion through the pore; this may be the reason for increase in percent hemolyis with temperature. The hemolysis of the erythrocytes in the presence of lectin showed a sharp rise with the increasing pH upto 7.5 and became constant till pH 9.5. This might reflect the presence of some ionizable group of amino acid residues of lectin with a pKa in the range of pH 7.5-9.5, which is involved in the hemolytic activity.

Presuming that the increased hemolysis of lectin agglutinated erythrocytes is due to the formation of ion permeable leaks formed in the membrane, as with some cytolytic proteins (Hatakayema et al., 1994; Hatakayema et al., 1995), attempts were made to prevent the colloid osmotic lyses by addition of non-electrolytes to the extra cellular medium, which may counter-balance the osmotic pressure exerted by intracellular macromolecules (Hoekstra and Duzgunes, 1989). The disaccharides like lactose and sucrose showed remarkable (85.2% and 77.8 % decrease, respectively with respect to hemolysis in PBS alone) osmotic protection against lysis, whereas, other sugars being low molecular weight molecules easily penetrated through the pores, thus, causing no effect. While lactose and sucrose being large weight carbohydrates could not pass through the leak, and therefore could protect the lysis maximally. In addition lactose may have also competed with lectin's active sites, thus, contributing more to the decrease in the lysis of erythrocytes.

The effect of lectin induced agglutination on the rate of oxidative damage to the cell membrane revealed that lectin induced perturbations in cell membrane makes it more vulnerable to oxidative attack. The leaks or pores formed in the lipid bilayer facilitated the superoxide ions to seep through the hydrophobic cellular membrane which was earlier impervious to these ions.

It is a well established fact that hypochlorous acid (HOCl) is an extremely toxic oxidant that can react with a variety of cellular components (Vissers and Winterbourn, 1991; Zavodnick et al., 2002). It has also been confirmed that HOCl causes red blood cell lysis through lipid modification, membrane cross linking, K^+ leakage and cell swelling (Vissers and Winterbourn, 1995; Zavodnick et al., 2002). Therefore, the effect of lectin on (HOCl) induced membrane perturbations was also studied. An additive effect on the increase in percent hemolysis of erythrocytes is observed by BBL on HOCl induced cell damage, thus, suggesting that lectin may be an active pore forming agent together with hypochlorite, thereby, making the membrane components more susceptible to oxidative assault. Brain lectin is a homodimeric protein with two

binding sites which can crosslink identical ligands on the cell surface or to the extracellular matrix inducing conformational changes in membrane proteins and altering lipid fluidity, and thus may increase the accessibility of HOCl to RBC components. As the average number of pores formed by HOCl per cell is less than 1.00 (Zavodnick et al., 2002), prior agglutination with lectin possibly increases the number of short lived pores in plasma membrane. This suggests the possibility of the role of β-galactoside binding lectin in HOCl induced neutrophil-mediated cellular damage, which may have direct implications in various inflammatory conditions of nervous tissues where increased expression of galectin has been reported (Rorive et al., 2001).

In addition, the peroxidant injury in any cell is greatly enhanced if its membrane integrity and fluidity is compromised (Hoekstra and Duzgunes, 1989). This has been confirmed as cells from pathological conditions with some membrane defects like sickle cell anaemia and thalassemia are more prone to oxidative assault than normal cells (Chin et al., 1982). Overall, our findings may also highlight the possible role of brain lectin as a triggering agent to enhance nervous tissue damage during oxidative stress by enabling the oxidant to penetrate inside cells due to the increased membrane permeability.

Thus, BBL exhibiting hemolytic and cytolytic functions might be involved in the defense mechanism of mammalian nervous system, not only neutralizing foreign substances by binding to their carbohydrate moieties, but also acting directly as a toxic protein to invading microorganisms. Thus, lectin's interaction with membranes acts as perturbing tool causing a change in membrane topology and assembly which may play important role under physiological demands or pathological conditions in mammalian nervous system.

The antigenic determinants present on the exterior surface of erythrocytes and other cell are carried by both glycolipids and glycoproteins, however; soluble blood group substances are strictly glycoprotein in nature (Carrilho et al., 2000; Connor et al., 2000; Hasija 2002; Hasija, 2004; Toivanen et al., 2008).

A number of glycoconjugates expressed on the erythrocyte and cell membrane are altered in primary cancerous and metastatic diseases. Alterations in serum glycoconjugates have been reported in patients with various cancers including head, oral and neck cancer (Baxi et al., 1991; Patel et al., 1997; Manoharan et al., 2004; Hernandez et al., 2006). Thus, altered glycoconjugates can be excellent indicators for

diagnosis, staging, prognostication, treatment monitoring and detecting early recurrence of cancer in patients with malignant neoplasm (Manoharan et al., 2004; Hasija, 2004; Hernandez et al., 2006). Lectins owing to their multivalent sugar binding property have been excellent tools for detection of aberrant glycosylation related to various carcinomas and may provide useful diagnostic or prognostic information, thus, contributing directly to cancer biology (Dennis et al., 1999; Hasija, 2004; Hernandez et al., 2006). Thus, β-galactoside binding lectin from buffalo brain was used to detect the change in the β-galactoside expression pattern in erythrocyte membrane from human donors suffering from prostrate and breast cancer. In case of erythrocytes from pre-operated breast cancer patients no change was observed in the hemolysis upon lectin binding, whereas, significant increase was observed in normal healthy controls and post-operated samples. This observation may be attributed to the decrease in the expression of β-1- 4 linked glycans on the surface of the RBC membrane in breast cancer patients, and their re-expression after surgery. Hemolysis of the lectin untreated RBCs from breast cancer patients displayed an increase as compared to the healthy controls owing to the pathological condition of the disease (Abou-Seif et al., 2000; Kolanjiappan et al., 2002). It has been observed that changes in binding qualities of blood group substances with lectins occur with the change in glycol-moieties of the glycoproteins (Hasija, 2004; Hernandez et al., 2006). Alterations in cell surface carbohydrates occur during malignancy which involves the blood antigens (Baxi et al., 1991; Hasija, 2004).

In contrast, an increase in hemolysis of BBL agglutinated RBCs was observed in prostrate cancer patients, suggesting no change in glycosylation with respect to BBL specific sugars in erythrocytes of these patients. As lectins or agglutinins are proteins that bind specifically to saccharide moieties present in glycoproteins or glycolipids on the cell surface without modifying them chemically (Nagae et al., 2008), they could be excellent tools to detect the changes in the glycosylation pattern during malignancy, which can be a more convenient method than taking the biopsy samples in certain cases (Hasija, 2002; Hasija, 2004).

To conclude, the purpose of the present work was not only to look for lectins, which display specific sugar specificity, but also to start a comprehensive study, which will contribute for the understanding biological functions of lectins.

Summary

SUMMARY

Depending on the requirement of calcium for their hemagglutination activity, the animal lectins have been divided into two major types, the S type (calcium-independent) and C type (calcium-dependent). The S-type lectins are soluble, β-galactoside-specific, evolutionarily conserved proteins widely distributed in nature from lower invertebrates to mammals. In addition, they require the presence of thiol reducing reagents to retain their carbohydrate-binding activity. So far, β-galactoside binding proteins or galectins have been implicated in various fundamental processes such as embryonic development, cell-cell interactions, cell migration, immune regulation and organization of nervous system. Moreover, these proteins are very well expressed in mammalian nervous system but its exact role is still obscure.

β-galactoside binding lectin from buffalo brain was purified to homogeneity by using a combination of 40-70% ammonium sulphate fractionation and gel filtration chromatography on sephadex $G_{50\text{-}80}$ column. The purification resulted in 1716 fold enrichment of specific activity with a yield of 0.04 %. The molecular weight of buffalo brain lectin (BBL) as determined by SDS-PAGE under reducing and non-reducing conditions was 14.5 kDa, however, with gel filtration under native conditions, it was 28.5 kDa, revealing the dimeric form of the protein. The stokes radius calculated from gel filtration data was 25 Å and diffusion coefficient corresponded to 8.91×10^{-15} cm^2/s.

The neutral sugar content of the soluble lectin was estimated to be 3.3 %, whereas thiol analysis indicated the presence of 3 sulphydryl groups per mole of BBL. Alkylation of sulphydryl groups of brain lectin by iodoacetate and iodoacetamide resulted in inactivation of hemagglutinating activity, suggesting the need of reducing agent to maintain BBL in its active form. The specificity of BBL for various saccharides was determined by hemagglutination inhibition assay. BBL displayed affinity for lactose and other sugar moieties in glycosidic form, suggesting it to be a β-galactoside binding lectin. Moreover, BBL preferentially agglutinated trypsinized human type A as well O erythrocytes. The temperature and pH activity profile displayed the maximum hemagglutination activity of BBL between a temperature range of 30-45 °C and a pH range of 7-7.5. Binding parameters of BBL for lactose was determined by equilibrium dialysis, with 6.6×10^3 M^{-1} association constant (K_{ass}) and two binding sites per lectin molecule.

The effect of various denaturing agents like GdnHCl, urea and thiourea on BBL was studied and it was found that BBL considerably lost its activity at 6 M concentration of denaturants. Similarly, SDS also abolished the lectin activity but at very low concentration, while non-ionic detergents like Tween-20 and Triton X-100 exhibited a mild inhibitory behavior against BBL. However, pre-incubation of BBL with lactose largely prevented the detergent induced denaturation of the protein.

The purified lectin was investigated for its brain cell aggregation properties by testing its ability to agglutinate cells isolated from buffalo and goat brains. Rate of aggregation of buffalo brain cells by purified protein was more than the goat brain cells.

The treatment of BBL with oxidizing agent (H_2O_2) abolished its agglutination activity and shifted its UV absorption maxima from 282 to 250 nm, suggesting the oxidation of tryptophan residue present in BBL. In addition, conformational changes in BBL induced by H_2O_2 were also monitored by fluorescence, circular dichroism and FTIR spectroscopy. Fluorescence spectra of BBL in presence of oxidizing agent displayed a quenching in the fluorescence intensity profile, confirming the oxidation of aromatic acid residues. However, the presence of lactose was protective against oxidative structural perturbations as the fluorescence intensity of BBL pre-incubated with lactose displayed a lesser degree of H_2O_2 induced quenching. Similarly, circular dichroism and FTIR analysis revealed that the presence of oxidizing agent completely destroyed the native conformation of BBL. Far UV and FTIR spectra of oxidized BBL suggested the loss of secondary state, thereby changing the β-pleated (native) into α-helical structures, whereas the near-UV spectra confirmed the oxidant induced perturbations in the native tertiary structure of BBL. However, pre-incubation of BBL with lactose did not bring any change in the native conformation of BBL both at secondary and tertiary levels. Moreover, a protective effect of lactose against oxidizing action of H_2O_2 on BBL was also demonstrated by both CD and FTIR spectroscopy.

The purified lectin from buffalo brain revealed the presence of 3.3 % sugar residues. Thus, the possible role of carbohydrate moiety in the stabilization of the protein was investigated by deglycosylating the lectin using periodate oxidation method. Deglycosylated and glycosylated forms of brain lectin were subjected to a comparative analysis using protein activity and fluorescence as probes over a wide range of temperature, pH and in the presence of various detergents and chaotropic

agents. The native form of lectin retained greater fraction of hemagglutinating activity against various physical and chemical denaturants. The unfolding of both the forms of lectin in the presence of GdnHCl, urea and thiourea studied by fluorescence indicated greater perturbations in the conformation of deglycosylated lectin than the native protein. The different properties examined thus indicated that glycosylation plays an important role in the stabilization of native conformation of protein against the inactivation caused by various denaturants.

Antibodies raised against pure BBL gave a single precipitin band with BBL and the titre determined by direct binding ELISA was found to be more than 12800 suggesting the high immunogenic nature of the protein. Moreover, anti-BBL antibodies also cross reacted with purified goat and sheep brain lectins, displaying antigenic relationship between them. Dot blot analysis revealed the presence of similar lectin in lung, heart and liver of buffalo which cross reacted with antibodies raised against buffalo brain lectin.

The effect of soluble β-galactoside specific lectin from buffalo brain on the fragility and permeability of bio-membranes was examined using erythrocyte as model. Osmotic fragility of erythrocytes was considerably enhanced in the presence of BBL. The lytic activity of BBL was temperature, pH and incubation period dependent with maximum activity displayed between 30-40°C, pH 6.4-7.5 and 6 hours, respectively.

Modulation of membrane integrity under oxidative stress in the presence and absence of β-galactoside binding protein was also investigated. Exogenous brain lectin considerably enhanced the susceptibility of erythrocyte membranes to free radical injury and hypochlorous acid induced oxidative assault, indicating the property of lectin to affect membrane dynamics and functions by overall reorganization of membrane components and making it more vulnerable to toxic assaults.

BBL was also used as a diagnostics tool to reveal the expression pattern of β-galactoside residues on the erythrocyte membranes of prostate and breast cancer patients. It was found that breast cancer causes a marked decrease in the expression of β-galactoside residues whereas, no change in the sugar moieties was exhibited in patients with prostate cancer.

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LIST OF PUBLICATIONS/PRESENTATIONS

- **Sabika Rizvi** and Naheed Banu, 'Physicochemical Properties and Oxidative Inactivation of Soluble Lectin from Water Buffalo (*Bubalus bubalis*) Brain', *Neurochem. Res.* (2008) 33:468–476.
- **Sabika Rizvi** and Naheed Banu , 'Prior treatment of erythrocytes with brain lectin increases its osmotic permeability and susceptibility to HOCL induced oxidative damage', (Manuscript communicated).
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Neurochem Res (2008) 33:468–476
DOI 10.1007/s11064-007-9456-0

ORIGINAL PAPER

Physicochemical Properties and Oxidative Inactivation of Soluble Lectin from Water Buffalo (*Bubalus bubalis*) Brain

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Accepted: 23 July 2007 / Published online: 31 August 2007

Abstract Lectins are carbohydrate-binding proteins present in a wide variety of plants and animals, which serve various important physiological functions. A soluble β-galactoside binding lectin has been isolated and purified to homogeneity from buffalo brain using ammonium sulphate precipitation (40–70%) and gel permeation chromatography on Sephadex G_{50-80} column. The molecular weight of buffalo brain lectin (BBL) as determined by SDS-PAGE under reducing and non-reducing conditions was 14.2 kDa, however, with gel filtration it was 28.5 kDa, revealing the dimeric form of protein. The neutral sugar content of the soluble lectin was estimated to be 3.3%. The BBL showed highest affinity for lactose and other sugar moieties in glycosidic form, suggesting it to be a β-galactoside binding lectin. The association constant for lactose binding as evidenced by Scatchard analysis was 6.6×10^3 M^{-1} showing two carbohydrate binding sites per lectin molecule. A total inhibition of lectin activity was observed by denaturants like guanidine HCl, thiourea and urea at 6 M concentration. The treatment of BBL with oxidizing agent destroyed its agglutination activity, abolished its fluorescence, and shifted its UV absorption maxima from 282 to 250 nm. The effect of H_2O_2 was greatly prevented by lactose indicating that BBL is more stable in the presence of its specific ligand. The purified lectin was investigated for its brain cell aggregation properties by testing its ability to agglutinate cells isolated from buffalo and goat brains. Rate of aggregation of buffalo brain cells by purified protein was more than the goat brain cells. The data from above study suggests that the isolated lectin may belong to the galectin-1 family but is glycosylated unlike those purified till date.

Keywords Buffalo brain lectin · Ammonium sulphate precipitation · Gel filtration · Oxidation · Fluorescence · UV spectra · Equilibrium dialysis · Brain cell aggregation

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Introduction

The galectins are a phylogenetically conserved family of animal lectins that recognize sugar residues present on cellular glycoproteins or glycolipids [1] and show a significant sequence similarity in their carbohydrate recognition domain (CRD). This class of proteins is widely distributed in the animal kingdom ranging from nematodes to human beings. Till date 15 members of galectin family have been identified in a variety of animal tissues like muscles, intestines, heart, lung and liver [2–4]. According to their structure they have been classified by Hirabayashi and Kasai [5] into prototype galectins (galectin-1, -2, -5, -7, -10, -11 and -13), existing as monomers or non-covalent homodimers, chimera type lectin (galectin-3) consisting of a non-lectin domain linked to a CRD, and tandem repeat type (galectin-4, -6, -8, -9 and -12) composed of two distinct CRDs in a single polypeptide chain. Unlike other proteins that are typically targeted to single cellular locations, galectins are found at many sites within and outside the cells. They are produced as cytosolic proteins, but can localize to the nucleus or associate with mitochondrial membranes [1]. In addition, galectin-9 can even insert in plasma membrane where it acts as a urate transporter in renal epithelium [6]. Apart from their cell adhesion potential, galectins also modulate cell proliferation

apoptosis, RNA splicing, inflammation, atherosclerosis and diabetic vascular nephropathy [7]. Thus galectins have an overwhelming variety of essential functions and serve as important biological tools.

In 1973 Rosen et al. first reported galectin expression in mammalian brain extract [8]. Since then galectins from mammalian nervous tissues have been an interesting target for active research and exploration. This galactoside-binding protein is localized in central nervous system in developing animals but its distribution is restricted to the peripheral nervous tissues in adults [9, 10]. In animal models galectin-1 (Gal-1) is expressed in a subpopulation of dorsal root ganglia neurons, primary sensory neurons, and motor neurons as well as astrocytes, perivascular cells and microvessels [10, 11]. Role of galectin in mammalian nervous tissue is little known. However, Gal-1 is involved in rodent olfactory system in neurite outgrowth and synaptic connectivity [12]. Recently, it has also been demonstrated that oxidized galectin enhances axonal regeneration in the rat dorsal root ganglion and may also contribute to the establishment of neuropathic pain after peripheral nerve injury [13]. By directly regulating the balance between cell survival and cell death, modulating cell motility, and impacting signalling through the Ras pathway, galectins may play important roles in the development and the potential treatment of brain tumours [14]. Although Gal-1 is widely expressed in mammalian brains but its exact function is still obscure. It has been purified and characterized from brain tissues of various mammals like rat, cow, human, goat, buffalo and sheep [15–19]. In view of the fact that the physicochemical properties of buffalo brain lectin (BBL) have not been fully investigated, we have initiated a detailed study of this soluble lectin, as it is available in large amounts, hoping that the intimate knowledge of this protein may help in understanding its physiological functions. In the present paper we have purified lectin having an affinity for β-galactosides from water buffalo (*Bubalus bubalis*) brain by gel permeation technique. Its detailed physicochemical properties have been sketched down and an attempt has been made to observe the changes in the function and structure of purified lectin in the presence of oxidative assault in order to elucidate the role of reducing agents to maintain its active form. Thus the overall aim of this work contributes to the existing repertoire of the diversity, structure, and biological role of brain lectins.

Materials and methods

Materials

Molecular weight marker proteins, trypsin, bisacrylamide, acrylamide, sephadex G_{100}, sephadex G_{50-80} were purchased from Sigma Chemical Co., St. Louis, MO, USA. Saccharides used were from Sisco Research Laboratories, Bombay, India. All other chemicals were of analytical grade. Fresh buffalo brain tissues were obtained from the local slaughterhouse and used immediately.

Methods

Purification of buffalo brain lectin (BBL)

Purification of lectin from buffalo brain was achieved by a slight modification of the earlier method [18]. Fresh nervous tissue (150 g) was properly cleaned, minced and homogenized in 75 mM phosphate buffered saline (PBS) pH 7.2 containing 0.15 M NaCl, 0.15 M lactose, 5 mM β-mercaptoethanol (β-ME), 0.02% (w/v) sodium azide and 10 mM EDTA. Homogenate was clarified by passing through several folds of cheesecloth and centrifugation at 10,000 rpm at 4°C. Supernatant was further sedimented at 40,000 rpm for 1 h to pellet out any insoluble matter. The brain extract (supernatant) was then fractionated between 40 and 70% ammonium sulphate saturation. The precipitated protein after dialysis against 75 mM PBS containing 40 mM lactose was applied on sephadex G_{50-80} column. The fractions of protein peak were again dialyzed against 75 mM PBS with 5 mM β-ME to remove lactose and tested for hemagglutination activity. Samples showing maximum activity were pooled and concentrated for study. The protein content was quantitated by Folin's phenol reagent by the method of Lowry et al. [20].

Determination of hemagglutination activity

The agglutination assays of BBL were performed with trypsinized rabbit erythrocytes to a final concentration of 100 μg/mL of RBC suspension according to the method of Lis and Sharon [21].

Hemagglutination activity was performed using 'U' shaped microtitre plates where twofold serial dilutions of 50 μL samples were made in 50 μL of operating buffer. RBC suspension in PBS (50 μL of 4% (v/v)) were added to each well, mixed by shaking and left at room temperature. The agglutinating titre (reciprocal of the highest dilution giving a visible hemagglutination) was noted after 1 h.

Carbohydrate specificity

Twofold serial dilutions of various sugars and their derivatives were made in 50 μL of operating buffer. Purified lectin solution (4 agglutinating unit) was added to each

well followed by the addition of 4% (v/v) trypsinized erythrocytes suspension. Agglutination was noted after 1 h. Highest dilution of the test sugars required for complete inhibition were recorded.

Molecular weight determination

SDS-PAGE of protein samples and molecular weight standards were performed in 12.5% acryl amide gel in both reducing and non-reducing conditions. Molecular weight of the native lectin was reconfirmed by size exclusion chromatography on sephadex G_{100} gel column. Standard molecular weight markers used were in the range of 96,000–14,500 Da. The linear plot between Ve/Vo and log M was used for calculating the molecular weight of the lectin. Stokes radius of the lectin was also determined using gel filtration data.

Chemical analysis

Carbohydrate content of the purified lectin was determined using the phenol sulphuric acid reaction described by Dubois et al. [22].

Effect of detergents and metal ions on lectin induced hemagglutination

The effect of denaturants on lectin activity was determined by incubating protein (125 μg/mL) with urea, guanidine HCl (GnHCl) and thiourea in the concentration range of 0.5–8.0 M. Lectin sample in PBS served as control. Titre value of each sample was determined using microplate assay.

To examine the effect of divalent cation on lectin activity demetallization of purified lectin was carried out using 0.1 M EDTA followed by remetallization of the sample with 0.1 M $CaCl_2$ and $MnCl_2$. Hemagglutination activity of each sample was tested using rabbit RBCs.

Equilibrium dialysis

The binding of lactose to BBL was quantitatively studied in 75 mM PBS pH 7.2 containing 0.15 M NaCl and 5 mM β-ME by equilibrium dialysis in a dialysis bag (3.0 mL capacity) made from Sigma cellulose membrane. The dialysis bag containing 100 μM of the lectin solution was placed in plastic vials containing 1.0 mL of lactose solution in the range of 40–400 μM. After equilibrating for 24 h at 37°C, a portion was taken from the protein free compartment and estimated for its carbohydrate content by the method of Dubois et al. [22]. The amount of lactose bound per mole of lectin was calculated according to Scatchard analysis.

Treatment of lectin with hydrogen peroxide

Lectin with a titre value of 256 was incubated with H_2O_2 (5 mM) in 75 mM PBS pH 7.2 in the absence of β-ME for 2 h at room temperature. Titre value of the incubated protein was determined.

Optical properties

Absorption spectroscopy. UV absorption spectra of native lectin in 75 mM PBS pH 7.2 containing 5 mM β-ME and oxidized lectin (by adding 5 mM H_2O_2) in the absence of β-ME was measured on Beckman DU-640 Spectrophotometer in the range of 220–320 nm.

Fluorescence spectroscopy. Intrinsic fluorescence of native protein was measured at 25°C in a Hitachi F-200 spectrofluorometer (Hitachi, Tokyo, Japan) equipped with a DR3 recorder. The protein was selectively irradiated using an excitation wavelength of 280 nm with 10 nm band pass. Emission spectra were measured in the range of 300–400 nm. Change in the intrinsic fluorescence in the presence of 5 mM of H_2O_2 and 0.1 M lactose solution was also recorded. Appropriate controls containing the oxidizing agent used for the treatment were run and correction made wherever necessary. Each spectrum was the average of three scans.

Brain cell aggregation assay

The brain cell aggregation activity was determined using dissociated cells from goat and buffalo brain without any chemical treatment. One-gram tissue was isolated from the telencephalon of each brain and mechanically dissolved into separate cells using a dissociation chamber containing a magnetic stirrer. The cell suspension was fixed in 4% (v/v) glutaraldehyde in 75 mM PBS pH 7.2 containing 0.15 M NaCl. After 1 h, the cells were washed in PBS, rinsed twice in 0.2 M glycine, and dissociated again. Isolated cells were finally suspended at 6.25×10^5 cells/mL of PBS pH 7.2 containing 75 mM NaCl. One hundred μL of purified lectin (10 μg/mL) was added to tubes containing 100 μL of a suspension of isolated fixed cells. After rotation at 70 rpm for 60 min, small aliquots were carefully removed and the number of particles (single cells and clumps) per unit volume was determined using a Maleuzz

hematocytometer. The rate of agglutination was expressed as the % decrease in particle number [23].

Brain cell aggregation activity of galectin was also measured as a function of pH and temperature by microtitre plate assay.

Results

Isolation of buffalo brain lectin

A lectin that displays affinity towards galactoside residues was purified by the combination of ammonium sulphate fractionation and gel permeation chromatography on Sephadex G_{50-80} column. Ammonium sulphate fractionation resulted in about 18% yield of the total active protein with fivefold purification. The high salt content was removed by extensive dialysis against 75 mM PBS pH 7.2 containing 30 mM lactose and 5 mM β-ME. Lactose was added to the dialyzed sample before loading on to the column to inhibit the lectin and Sephadex gel interaction. The yield of the purified protein was around 560 μg (from 1,350 mg) with fold purification 1,715.59 (Table 1). Upon SDS-PAGE of the fraction showing the maximum activity, a single band was observed, suggesting homogeneity of the preparation (Fig. 1a).

Molecular weight determination

The lectin purified from buffalo brain gave a single polypeptide band corresponding to ~14.5 kDa as assessed by SDS-PAGE under reducing and non-reducing conditions (Fig. 1b). This result was on the basis of three independent SDS-PAGE studies. Using size exclusion chromatography, the native protein appeared as a single peak corresponding to 28.5 kDa, suggesting that it is composed of two identical non-covalently linked 14.5 kDa subunits. The stokes radius computed using the gel filtration data was found to be ~25 A° (data not provided).

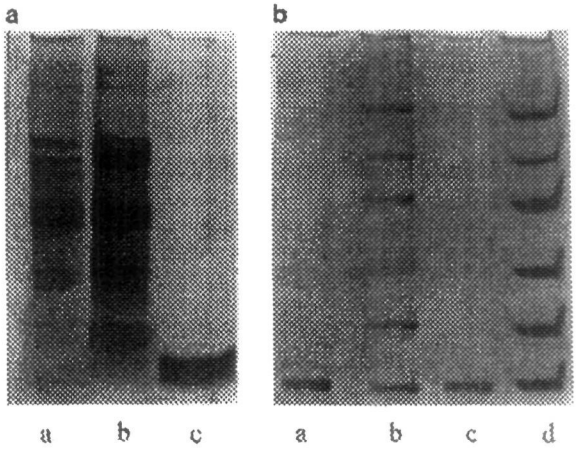

Fig. 1 **a** SDS-Gel electrophoresis of buffalo brain lectin during various stages of purification: SDS-PAGE was performed on 12.5% acrylamide gel under reducing conditions. Lane (a) soluble brain extracts (30 μg); Lane (b) 40–70% ammonium sulphate fraction (30 μg); Lane (c) purified protein (40 μg). **b** Molecular weight determination of BBL: samples were separated by 12.5% SDS-PAGE under reducing condition and non-reducing conditions. Lane (a) purified protein (25 μg) under reducing conditions; Lane (b) molecular weight markers under reducing conditions; Lane (c) purified protein (25 μg) under non-reducing conditions; Lane (d) molecular weight markers under non-reducing conditions. The molecular weight markers are in the descending order; Phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Soyabean trypsin inhibitor (20.1kDa), Lysozyme (14.4 kDa)

Agglutination properties and stability of buffalo brain lectin

Some agglutination properties of the pure lectin towards trypsinized erythrocytes have been investigated. The extracted lectin showed maximum activity only in the presence of a reducing agent like 5 mM β mercaptoethanol and 4 mM dithiothreitol. The hemagglutination titre was not affected up to a temperature range of 45°C, and the most suitable pH for the protein to remain in active conformation is in the range of pH 6.0–9.0. Purified lectin showed no detectable loss of activity when kept for up to 3 months in PBS containing 0.3 M lactose and 5 mM

Table 1 Purification table of Buffalo brain lectin

Step fraction	Total volume (ml)	Total protein (mg)[a]	Total activity (titre)[b]	Specific activity (titre/mg)	Purification (fold)	Recovery (%)	Yield (%)
Brain extract	180	1350	14400	10.66	1	100	100
40–70% cut	20	240	12800	53.33	5.00	88.88	17.77
Gel chromatography	2	0.560	10240	18285.71	1715.35	71.11	0.041

Values are means of three different preparations from 150 g fresh tissue

[a] Determined by the method of Lowry et al

[b] The titre of the tested lectin is expressed as the reciprocal of the highest dilution showing agglutination of trypsinized rabbit erythrocytes

β-ME at 4°C. Carbohydrate specificity of the BBL was determined at room temperature by the hemagglutination inhibition assay. Lactose was found to be the most potent inhibitor of hemagglutination activity giving complete inhibition at 0.78 mM followed by *p*-nitrophenyl-β-D-galactopyranoside at concentration of 50 mM suggesting that the active sites of the lectin have high specificity for carbohydrates with glycosidic linkages (Table 2).

Effect of denaturants and metal ions

Denaturants like urea and GnHCl at 3.0 M concentration while thiourea at 4.0 M reduced the lectin activity to 50%. The denaturation of lectin was also monitored by fluorescence spectroscopy at 340 nm (Fig. 2) in the presence of increasing concentration of GnHCl. Complete unfolding of protein was observed at 6 M GnHCl with a 40% decrease in fluorescence intensity and shift of emission maximum from 340 to 357 nm. EDTA treatment or addition of metal cations showed no effect on lectin activity suggesting that lectin activity was not dependent on metal ions.

Chemical analysis

Sugar analyses performed on samples of lectin reveal that lectin contains 3.3% carbohydrate, which would correspond to four residues of carbohydrate per lectin molecule. To confirm the glycosylated form of BBL, it was assured that sugar residues were not contaminants remaining from the gel chromatography or originating from the dialysis bag since no carbohydrate could be detected in the sample of goat brain lectin purified under the same conditions as BBL.

Table 2 Effect of various sacchrides on the hemagglutinating activity of buffalo brain lectin

Carbohydrates	Minimum concentration of sugar giving complete hemagglutination inhibition
Lactose (100 mM)	0.78
Galactose (200 mM)	100
Methyl-β-D-galactopyranoside (200 mM)	100
p-nitrophenyl-β-D-galactopyranoside (200 mM)	50
p-nitrophenyl-α-D-galactopyranoside (200 mM)	Nil
D-galactosamine (200 mM)	>100
Methyl-α-D-galactopyranoside (200 mM)	100
Glucose (200 mM)	Nil

The following sacchrides were also tested and had no inhibitory activity at 200 mM: *p*-nitrophenyl-α-D-galactopyranoside, D-mannose, L-fucose, sucrose, melibiose, and D-fructose

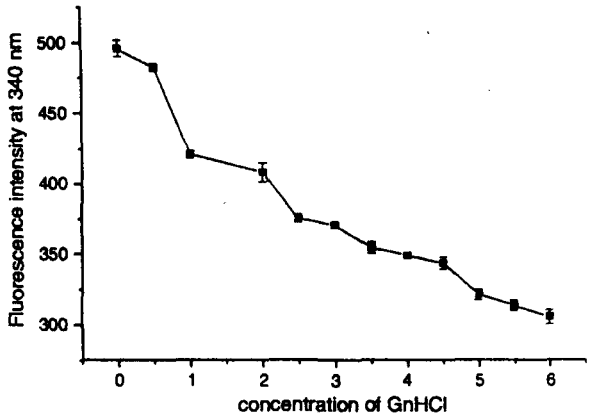

Fig. 2 Effect of growing concentrations of GnHCl on fluorescence intensity of native BBL at 340 nm. Protein concentration was 125 μg/mL in 75 mM PBS, 0.15 M NaCl, pH 7.2 containing 5 mM βME. The denaturation was monitored by fluorescence intensity at 340 nm

Equilibrium dialysis

The Scatchard plot of the equilibrium dialysis data of BBL in 75 mM PBS buffer containing 0.15 M NaCl and 5 mM β-ME (pH 7.2) with lactose at 37°C is shown in Fig. 3. A linear plot was obtained, the slope of which gave the binding constant K_{ass} as 6.66×10^3 M^{-1}. The number of binding sites calculated from the X intercept was found to be 1.7 per lectin dimer suggesting the presence of two binding site per protein molecule.

Spectroscopic properties of lectin

Purified protein presents a UV spectrum (Fig. 4) with maxima at 282 nm corresponding to the presence of single tryptophan residue and a large number of other aromatic residues. When excited at 280 nm, the native lectin shows a fluorescence emission spectrum with a maximum at 335 nm, typical of that of a tryptophan group in a hydrophobic environment as shown in Fig. 5.

Functional and conformational modification of lectin in the presence of H_2O_2 and lactose

H_2O_2 caused a remarkable decrease in hemagglutination activity of buffalo brain lectin with time. Activity was completely abolished after 40 min. The presence of an

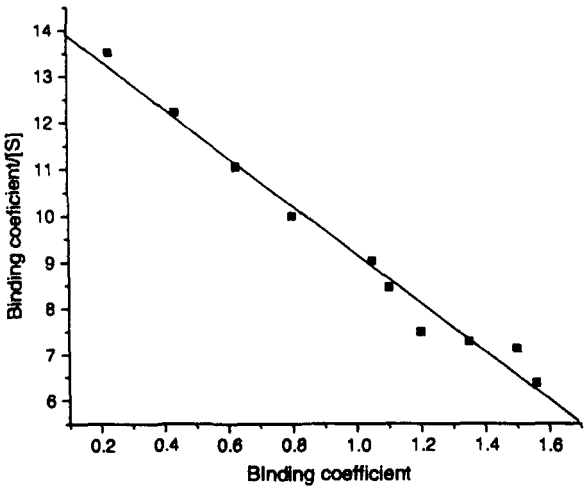

Fig. 3 Scatchard plot for the binding of lactose to BBL. A fixed concentration of BBL (100 μM) in 75 mM phosphate buffer pH 7.2 containing 0.15 M NaCl in a dialysis bag was incubated with 40–400 μM of lactose in the same buffer at 37°C for 24 h. After the attainment of equilibrium the decrease in lactose concentration was estimated in the dialysate. Analysis of results yielded the value of association constant and the number of sugar binding sites

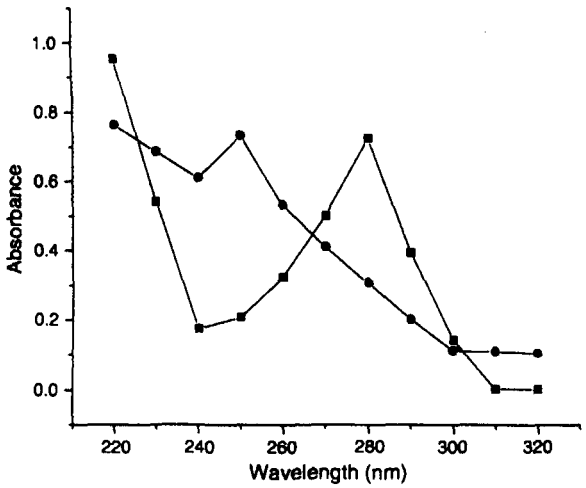

Fig. 4 Ultraviolet spectra of native and oxidized buffalo brain lectin. Spectra of native buffalo brain lectin (150 μg/mL) in 75 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl and 5 mM β-mercaptoethanol (■) and after adding 5 mM H_2O_2 in absence of β-mercaptoethanol (●)

oxidant also shifted the absorption maxima of BBL from 282 to 250 nm (Fig. 4). The fluorescent profile of the oxidized protein (Fig. 5) showed a remarkable quenching in the fluorescence intensity accompanied with a blue shift from 335 to 342 nm. When fluorescence experiments of the native protein were carried out in the presence of 0.1 lactose solution a large enhancement in the fluorescence

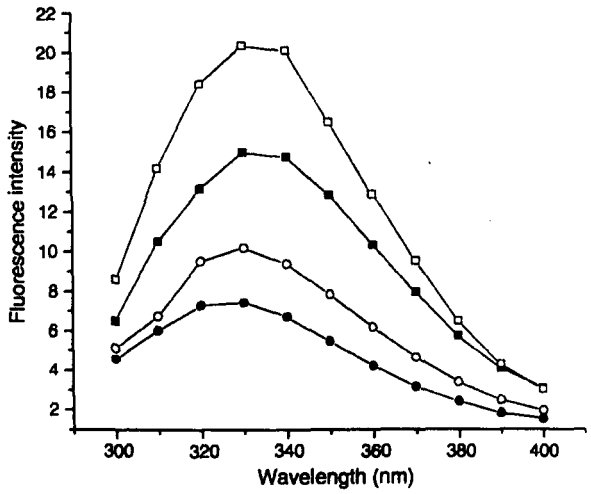

Fig. 5 Fluorescence spectra of native and oxidized buffalo brain lectin. Experiments were performed with BBL (45 μg/mL) in 75 mM sodium phosphate buffer pH 7.2. The spectra of native BBL alone (■), in the presence of 0.1 M lactose (□), oxidized BBL alone (by adding 5 mM H_2O_2) (●) and oxidized BBL in the presence of 0.1 M lactose (o) were measured at 300–400 nm

intensity was observed. Moreover, exposure of lectin to 5 mM H_2O_2 in the presence of 0.1 M lactose showed less decrease of fluorescence intensity as compared to the decrease in the absence of lactose solution.

Brain aggregation assays

The purified BBL preferentially agglutinated buffalo brain cells (75%; 45 × 10^4 cells/mL) than goat brain cells (63%; 38 × 10^4 cells/mL) (Fig. 6). Optimum pH and temperature for brain cell aggregation activity of BBL was found to be 7.5 and 40°C, respectively.

Discussion

In this study we have described the purification of lectin from the nervous tissue of buffalo on the basis of gel filtration technique. The present rapid and simple scheme of purification involving ammonium sulphate precipitation and gel filtration chromatography on Sephadex G_{50-80} column gave a better yield as compared to our earlier method [18]. The lectin was electrophoretically homogenous in SDS-PAGE and migrated as a single band corresponding to a molecular weight of 14.5 kDa similar to the lectin isolated from bovine, rat and human nervous tissue [15, 16]. In size exclusion chromatography, the native lectin behaved as a globular protein with molecular weight of 28.5 kDa suggesting that it is presumably

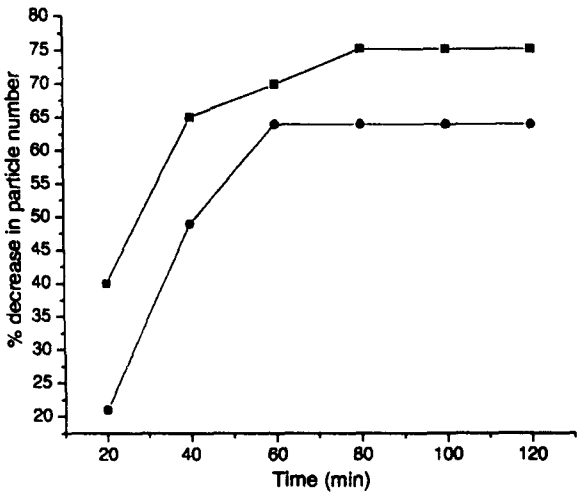

Fig. 6 Brain cell aggregation by BBL. Lectin induced aggregation of brain cells from adult buffalo (■) and goat (●) were detected by measuring the decrease in the number of total free particles by hematocytometer

composed of two identical subunits. These results were consistent with the values obtained for the lectin from nervous tissue of other mammalian species [15, 16, 24]. The dimeric structure was further confirmed by the Scatchard analysis, which indicated the presence of two carbohydrate binding sites per native molecule of lectin. This result is quite comparable to that obtained for bovine spleen galectin [25]. Association constant (K_{ass}) for the binding of lactose to BBL was $\sim 6.66 \times 10^3\ M^{-1}$, which is slightly lower than that of electrolectin [26]. The purified lectin is a glycoprotein containing 3.3% carbohydrate unlike other brain lectins purified in our laboratory [17–19]. This sheds light on the significance of glycosylation, which is one of the most important posttranslational modifications for newly synthesized proteins [27]. BBL being a glycosylated protein opens further avenues for future studies in understanding the role of glycosylation in protein function. Lectin obtained from buffalo brain was maximally inhibited by lactose, suggesting that it has an affinity for carbohydrate moieties in glycosidic forms. Gal-1 is a non-covalent homodimeric protein composed of 14.5 kDa subunits consisting of two identical conserved CRDs [7]. Thus BBL isolated in the present study is a β-galactoside binding protein resembling the Gal-1 family in functional and structural respects, but inclusion of this lectin in the Gal-1 family still awaits the sequence analysis for the presence of sequence similarity in CRD domains. The spectroscopic analysis of BBL showed that lectin fluoresces at 335 nm and its UV maxima are 282 nm confirming the presence of aromatic residues particularly tryptophan moiety in a hydrophobic environment. The denaturation of BBL by various denaturants indicated its globular nature stabilized mainly by hydrogen binding and hydrophobic interactions [28]. The fluorescence emission spectrum of BBL in the presence of increasing concentrations of GnHCl showed a decrease in the fluorescence intensity indicating that the tryptophan residue in native protein is located near an intra-molecular quenching group and the denaturation of the protein increases this interaction. These changes were also accompanied by a red shift from 340 to 357 nm at different concentrations of GnHCl. Higher denaturant concentrations, up to 6 M GnHCl, had no effect on the position of the maximum. This shift results from the extent of exposure of the Trp residue to bulk solvent. In all, these data show that BBL undergoes a guanidine-induced alteration in its conformation that involves a modification of the environment of the Trp residues [29].

Nearly all known galectins with few exceptions require reducing agents for their activity [30]. Buffalo brain lectin also shows hemagglutination activity only in the presence of reducing agents such as β-ME. Stability studies also demonstrated that lactose maintains the purified protein in the active form even in the absence of a reducing agent, possibly by preventing oxidative inactivation due to formation of intra-sulphide linkages [31]. Since these lectins remain in active conformation in the presence of a reducing agent, it led us to believe that they contain an oxidizable, residue whose integrity is quite crucial for its activity. The exposure of lectin to an oxidant causes a decrease in its activity, which can be due to the oxidation of either cysteine or tryptophan residue. Oxidation of cysteine residue can be possibly ruled out as UV absorption maxima in the presence of an oxidant showed a sharp decline from 282 to 250 nm, typical to that of an oxidized tryptophan residue [32]. It was further confirmed by quenching of the intrinsic fluorescence of the protein in the presence of 5 mM H_2O_2 with a slight blue shift. Apparently, H_2O_2 did induce subtle changes in lectin conformation in such a way that fluorophor is transferred to less polar environment and also led to the oxidation of tryptophan residue to form an oxindole. The findings that lactose increases the fluorescence of lectin and prevents the deleterious effect of H_2O_2 indicate that the emitting fluorophore is located within the lactose-binding site [26]. Since the tryptophan fluorescence is quenched by H_2O_2, the loss of lectin activity can be accounted for by the oxidation of the tryptophan residue present in the lactose-binding site. In the presence of lactose, oxidation of the tryphtophan residue and formation of oxindole is prevented. Thus these findings suggest the importance of reducing environment which is probably needed to reduce molecular oxygen normally present in solution and prevent it from oxidizing tryptophans [26]. Thus, due to high susceptibility of brain lectins to oxidation they are often advised to store in lactose solution.

The exact biological action of 14-kDa lectin in brain has not yet been known. Recent evidence indicates that the brain lectins are involved in the potentiation of neuropathic pain in the dorsal horn of rat olfactory system [12]. However, it has been demonstrated that oxidized galectins also enhances axonal regeneration in the peripheral nervous system but does not posses lectin properties [33]. Our studies also demonstrated that oxidized form is totally inactive thus proving that oxidized functional lectin in the nervous tissue may just acts as an autocrine or paracrine factor, functioning more like a cytokine or chemokine than as lectin. Thus this information opens new insights into the functions of galectins in mammalian nervous system.

Interestingly, our findings also showed that mammalian galectins are potent inducers of mammalian brain cell aggregation. It is important to note that galectin carbohydrate binding need not be the only, or even the main determinant of specificity in brain cell interaction. A requirement for dual recognition involving other sets of complementary molecules is consistent with the lectin recognition hypothesis [23]. This result may also imply the involvement of the brain galectins in neurotransmission process and neuronal cell differentiation.

To conclude, the purpose of this study was not only to look for lectins, which display specific sugar specificity, but also to start a comprehensive study, which will contribute for the understanding biological functions of lectins.

Acknowledgements This work was supported by a grant from Council of Science & Technology, UP, India. The laboratory facilities provided by UGC-DRS, DST-FIST and Aligarh Muslim University, Aligarh, are thankfully acknowledged.

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